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Most biofilms involved in catheter-associated urinary tract infections (CAUTIs) are polymicrobial, with disease causing (eg *Escherichia coli*) and atypical microorganisms (eg *Delftia tsuruhatensis*) frequently 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* and *Achromobacter xylosoxidans*), were evaluated. All species were good biofilm producers (Log 5.84–7.25 CFU cm^{-2} at 192 h) in artificial urine. The ability of atypical species to form a biofilm appeared 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. The 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.

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

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Introduction

Hospital-acquired (nosocomial) infections are frequently related to biofilms formed in medical devices, such as prosthetic heart valves, cardiac pacemakers, urinary catheters, contact lenses and orthopedic devices (Morris & Stickler 1998; Hall-Stoodley et al. 2004; Campoccia et al. 2006; Tenke et al. 2006; Silva et al. 2010). The higher economic costs associated with these diseases is due to long hospitalization periods for infected patients (Ferrieres et al. 2007; Curtis 2008; 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 (Doyle et al. 2001; Curtis 2008). 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, infection is inevitable in most 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 (eg antibodies, and phagocytes) or other environmentally-challenging conditions (eg UV light, extreme temperatures, and shear forces) (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 (Macleod & Stickler 2007; Frank et al. 2009; Hola et al. 2010). 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 conjunction with initial colonizers (Matsukawa et al. 2005; Jacobsen et al. 2008). Furthermore, it was recently observed that these disease causing microorganisms can co-inhabit the catheter surface with other unusual microorganisms with unproven pathogenic potential (eg *Delftia*

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tsuruhatensis and *Achromobacter xylosoxidans*) (Frank et al. 2009). While interactions of *E. coli* with other common causes of UTIs have already been addressed (Matsukawa et al. 2005; Ferrieres et al. 2007; Jacobsen et al. 2008; Cerqueira et al. 2013); there is a lack of knowledge about the possible role that these atypical microorganisms play in the rate at which disease-causing microorganisms adhere and form biofilms and, consequently, their effect on the outcome of CAUTIs. Indeed, some studies have demonstrated recently, for other pathologies, that the atypical microorganisms could have important contributions in biofilm infections (Lopes et al. 2012, 2014).

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 (eg *E. coli*) and, eventually, they could act as primary colonizers of the catheter. On the other hand, it might be possible that the colonization by these atypical microorganisms can prevent colonization by pathogenic bacteria. Hence, understanding the role of atypical microorganisms in biofilm dynamics might be crucial to help in the development of novel strategies to prevent or minimize bacterial adhesion to catheters.

As such, in this study 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 was evaluated. To better mimic conditions found in urinary catheters, biofilms were formed in an artificial urine medium (AUM) (Brooks & Keevil 1997) at 37°C. In order to understand which type of interactions occur between different species, dual-species biofilms were compared with the fitness of individual biofilm regarding: the total biomass formed, total cell counts and cultivability values. Four additional features were also explored: the growth rates of each microorganism, 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 and *D. tsuruhatensis* BM90 were streaked from a frozen stock (–80°C) on Tryptic Soy Agar (TSA) (Merck, Darmstadt, 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 h) at 37°C, under agitation (150 rpm). The cell concentration was then assessed by optical density (OD) at 620 nm, and the inoculum was diluted in AUM in order to obtain a final concentration of 10^8 CFU ml^{-1} or 10^2 CFU ml^{-1}. AUM was prepared as previously described (Brooks & Keevil 1997), using the following formulation in 1 l of distilled water: peptone 1 g (Merck), yeast extract 0.05 g (Liofilchem, Roseto degli Abruzzi, Italy), lactic acid 1.1 mmol l^{-1} (Fluka, Portugal), citric acid 0.4 g (VWR, Leuven, Belgium), sodium bicarbonate 2.1 g (Merck), urea 10 g (VWR), uric acid 0.07 g (VWR), creatinine 0.8 g (Merck), calcium chloride.$2H_2O$ 0.37 g (Merck), sodium chloride 5.2 g (Merck), iron II sulfate.$7H_2O$ 0.0012 g (Merck), magnesium sulfate.$7H_2O$ 0.49 g (Merck), sodium sulfate.$10H_2O$ 3.2 g (Merck), potassium dihydrogen phosphate 0.95 g (Merck), di-potassium hydrogen phosphate 1.2 g (Merck) and ammonium chloride 1.3 g (Merck) (the 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 CFU 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 CFU ml^{-1} was performed in order to evaluate the influence of initial inoculation level on biofilm formation by the three species under study (the results are presented in Supplemental material) [Supplemental material is available *via* a multimedia link on the online article webpage].

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

Tissue culture plates were then placed in an incubator (FOC 225I; VELP Scientifica, Usmate, Italy) at 37°C, under static conditions, for 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

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as follows. (i) Pre-colonization with atypical microorganisms: wells of a 96-well tissue culture plate were pre-colonized with atypical microorganisms (at an initial concentration of 10^8 CFU ml^{-1}). After 24 h, the medium was removed, the 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 CFU ml^{-1}) were added. The same assay was performed but with initial concentrations of 10^2 CFU ml^{-1} for the atypical microorganisms and 10^8 CFU 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 a low initial concentration (10^2 CFU ml^{-1}). These assays were used as controls to compare the results obtained in biofilm experiments (i) and (ii).

At selected time points (24, 48, 96 and 192 h), formation of single and dual-species biofilms was assessed by crystal violet (CV) staining (for quantification of biomass formed), counts of colony forming units (CFUs) (for cultivable cell counts) and DAPI (4′-6-diamidino-2-phenylindole) staining (for total cell 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. The biofilm was sonicated for 4 min (70 W, 35 kHz, Ultrasonic Bath T420, Elma, Singen, 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 better discrimination between the two species. MacConkey agar (Liofilchem) 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 slow growth in this medium, but were easily distinguished due to their non-lactose fermenting phenotype. The other media used included: Cetrimide agar (Liofilchem) for *A. xylodoxidans* and Simmons Citrate agar (ammonium dihydrogen phosphate 1 g l^{-1} [Merck]; di-potassium hydrogen phosphate 1 g l^{-1} [Merck]; sodium chloride 5 g l^{-1} [Merck]; tri-sodium citrate 2 g l^{-1} [Sigma, St Louis, MO, USA]; magnesium sulfate 0.2 g l^{-1} [Merck]; bromothymol blue 0.08 g l^{-1} [Sigma]; agar 13 g l^{-1} [Merck]) for *D. tsuruhatensis.* Neither of these two media was able to recover *E. coli* cells. Afterwards, selective agar plates were incubated at 37°C for 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 were found between the CFU counts in TSA and in the selective/differential media used (data not shown).

Biomass quantification by the CV assay

The 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, the ethanol was removed and plates were allowed to air-dry. Then fixed biofilms were stained with 250 µl of CV (Merck) for 5 min. The wells were 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). Finally, plates were placed in agitation up to 2 min and the OD measured at 570 nm using a microtiter plate reader (Spectra Max M2, Molecular Devices, Sunnyvale, CA, USA).

DAPI staining

To assess total bacterial cell counts in single-species 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, Maidstone, UK). Subsequently the membrane was stained with DAPI (0.2 mg ml^{-1}) (Merck) and left for 10 min in the dark. Then, the membrane was placed on a microscope slide. Finally, a drop of immersion oil (Merck) was added and the membrane covered with a coverslip. Cells were analyzed using a Leica DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems, Wetzlar, Germany). The optical filter combination for optimal viewing of stained preparations (Chroma 61,000-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 h). Subsequently, cells were diluted in order to obtain a final OD (620 nm) of 0.1, incubated at 37°C, and 150 rpm. The OD at 620 nm was measured at different time points until the stationary stage was reached.

Siderophore 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 (1987). Following incubation at 37°C for 24 h, plates were analyzed for the presence of growth and orange halos.

Antimicrobial activity of biofilm supernatants

The presence of antimicrobial activity in 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, Maia, 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 μls of each supernatant was applied onto the lawns, and left to air-dry. Afterwards, the plates were incubated for 24 h at 37°C. The formation of halos was 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,

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

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

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

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 h 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 (Statistical Package for the Social Sciences, Chicago, IL, 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 CAUTI-associated biofilms often involve more than one microbial species, causing what can be defined as a polymicrobial disease (Macleod & Stickler 2007; Frank et al. 2009; Hola et al. 2010). As *E. coli* is one of the main pathogens involved in these infections (Svanborg & Godaly 1997; Hedlund et al. 2001; Ronald 2002; Niveditha et al. 2012), it would be expected that the biofilm-forming ability of this microorganism would surpass those exhibited by atypical microorganisms. The assessment of the bacterial growth rate in AUM has shown that the 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 (Figure S1). Higher biomass values were observed for *A. xylosoxidans*, which reached an OD of ~5 at 192 h, when compared with *D. tsuruhatensis* and *E. coli* (OD ~1.4 and ~2.4 at 192 h, respectively). Moreover, for *A. xylosoxidans* values, this biomass difference was statistically significant at 192 h ($p < 0.05$).

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 CFU 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}) (Figure S1) and, as expected, the CFU counts were always lower than the DAPI counts. In general, the averages of cells detected by cultivability

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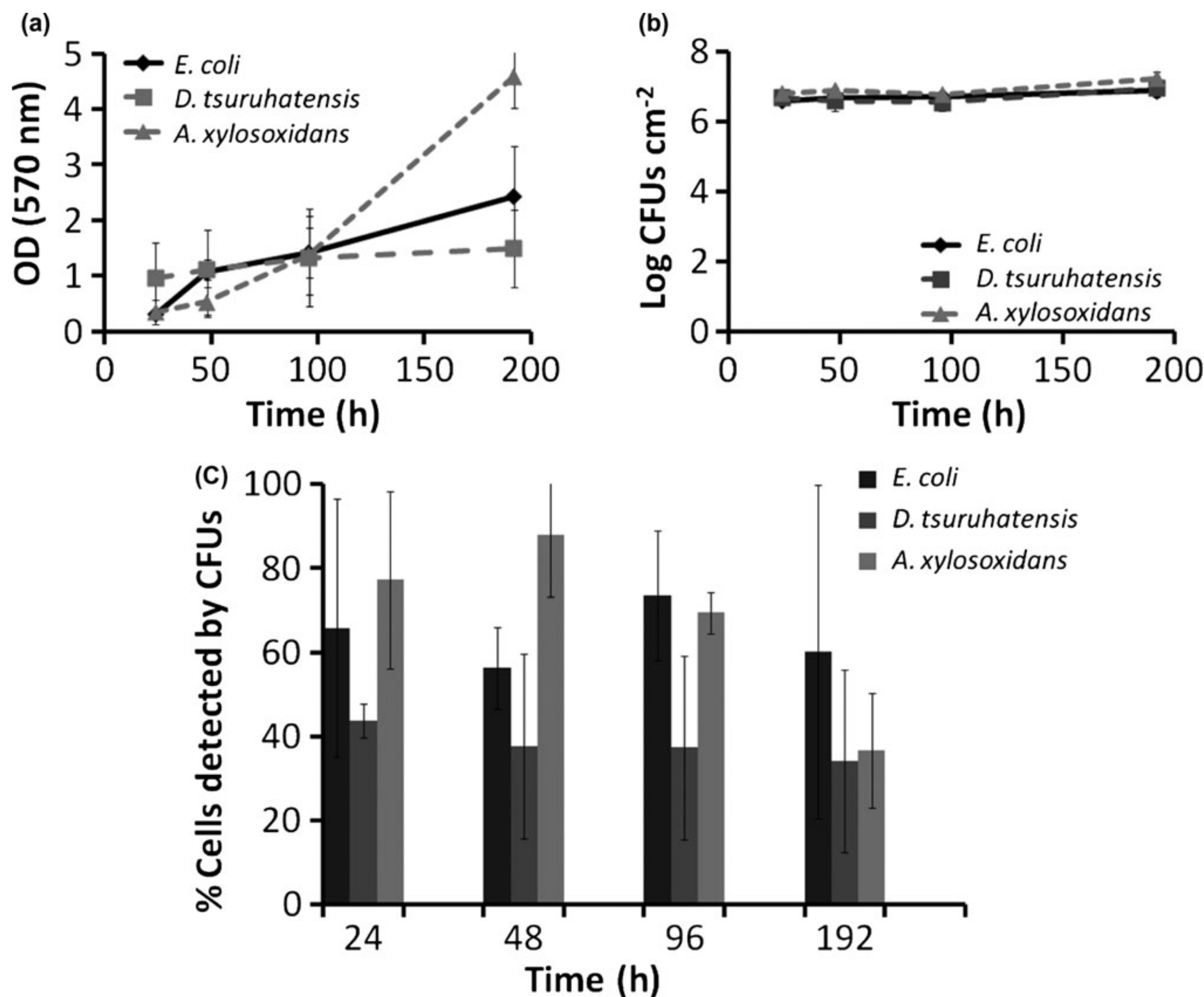

Figure 1. Biofilm formation for single-species biofilms. Values for (a) total biomass; (b) cultivability; and (c) percentages of cells detected by cultivability are presented for all species. Three independent experiments were performed for each condition. Error bars represent the SD.

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(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 *vs* 36.6% at 192 h, $p > 0.05$). For *D. tsuruhatensis*, the percentages obtained were always lower than those observed for 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 (Figure S2) and visualize the influence of the atypical species on *E. coli* biofilm formation, the relative fitness of *E. coli* in dual-species biofilms (Figure 2) was determined. In the presence of *D. tsuruhatensis* and *A. xylosoxidans*, the fitness of *E. coli* slightly increased over the time, reaching a value of 1.12 ($p < 0.05$) and 1.07, respectively, after 192 h. These conclusions can be observed in more detail in the CV and cultivability graphs (Figure S2). CV assays for dual-species biofilms showed that when *E. coli* is co-cultured with the atypical microorganisms the total biomass profiles tended to be more similar to that of *E. coli* single-species biofilm (Figure S2a, b). The data for cultivability assays in dual-species biofilms confirm that the ability of *E. coli* 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 (Figure S3) and, as expected, the CFU counts were always lower than the DAPI counts. However, it should be mentioned that the observations described here might be limited to the inoculum concentrations used in this study. To clarify this point, the influence of the

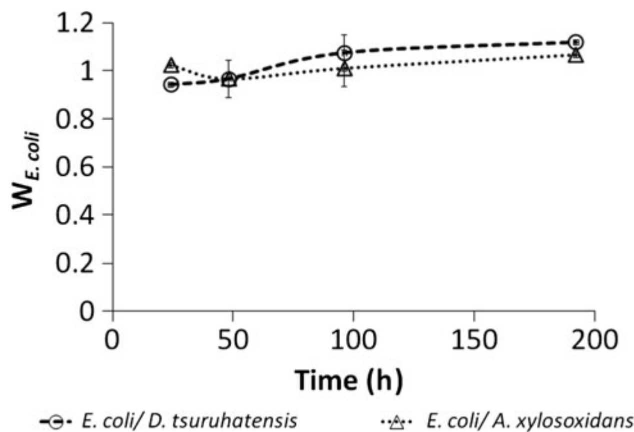

Figure 2. Relative fitness of *E. coli* in dual-species biofilms. Fitness of *E. coli* was determined in the presence of atypical species (*D. tsuruhatensis* and *A. xylosoxidans*) with simultaneous addition of the bacteria at the same initial concentration (10^8 CFU ml^{-1}). Data are means of three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

initial inoculation level (10^6 CFU ml^{-1} *vs* 10^8 CFU ml^{-1}) on biofilm formation was evaluated (Figure S4). No significant differences were found for *E. coli* ($p > 0.05$ for each point), which indicates that the initial inoculum concentration did not seem to have an influence on *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 CFU ml^{-1}, and lower cultivability values were observed for up to 24 h and up to 48 h, respectively, reflecting a delay in their biofilm formation. However, it does not affect the final biofilm concentration, which reached the same values (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) (Harrison 2007; Hibbing et al. 2010; Elias & Banin 2012). In order to explain the possible interaction between *E. coli* and atypical species in dual-species biofilm, four additional features were analyzed: the antimicrobial activity of biofilm supernatants in single and dual-species biofilms, siderophore production, the growth rate of each species, and the effect of a pre-formed biofilm on *E. coli* biofilm formation.

Antimicrobial activity of biofilm supernatants and siderophore production

An important factor in determining the dominant species within a mixed biofilm is the production of antimicrobial compounds, which might provide an advantage to the producer species by interfering or killing the neighboring microorganisms (Hibbing et al. 2010). However, in the present work, the examination of antimicrobial compounds in biofilm supernatants, either from single or dual-species biofilms, suggested that none of the microorganisms secreted compounds clearly able to influence the growth of the others. It might be possible that antimicrobial compounds are present at very low concentrations, as usually happens for the most part in the case of secondary metabolites; which would also appear as a negative result. Also, some other molecules that interfere with non-essential processes (eg quorum sensing molecules), are not detected in this type of assay. Nonetheless, the complete absence of any inhibitory signal suggests that the observed decrease in 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 iron sequestration by the production, release and uptake of siderophores (Griffin et al. 2004; Weaver & Kolter 2004; Joshi et al. 2006; Smith et al. 2006; Hibbing et al. 2010). Siderophores are molecules secreted under low iron availability and are used by microorganisms to sequester the iron available in the medium (Ratledge & Dover 2000; Andrews et al. 2003; Hibbing et al. 2010). The importance of iron acquisition has been reported for the survival of uropathogenic *E. coli* during CAUTIs development (Snyder et al. 2004; Jacobsen et al. 2008). Considering the low iron concentration in urine and its importance for microorganism growth and survival during CAUTIs (Shand et al. 1985; Jacobsen et al. 2008), this nutrient is expected to be consumed by microorganisms with a high ability to produce or utilize siderophores, limiting it to the other microorganisms. The 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 iron molecules, providing this microorganism with an advantage in iron-depleted conditions, such as during 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

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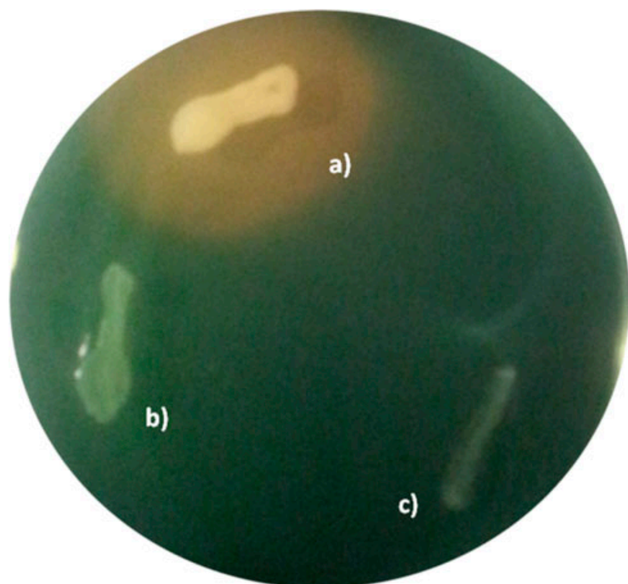

Figure 3. Screening for siderophore production using CAS agar plates. (a) On CAS agar, an orange halo surrounding the colony indicates that *E. coli* produces high levels of siderophores. The presence of growth without an orange halo indicates that (b) *D. tusuruhatensis* and (c) *A. xylosoxidans* produce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

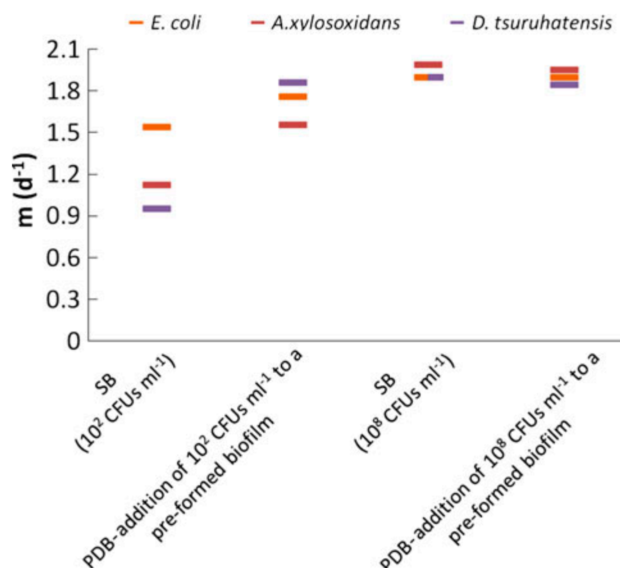

Figure 4. Values of the malthusian parameter for pre-colonization experiments. The malthusian parameter of the second microorganism added to a pre-formed biofilm was determined between time 0 and 48 h. Values of the malthusian parameter for single-species biofilm of each microorganism were determined for comparative purposes. SB = single-species biofilm; PDB = pre-colonization dual-species biofilm.

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experiments were performed with different inoculum concentrations (10^8 CFU ml^{-1} and 10^2 CFU ml^{-1}) to determine whether the inoculation level influenced the adhesion of a second species to the biofilm.

The addition of *E. coli* to a pre-formed biofilm did not lead to significant changes in the total biomass compared to experiments with synchronized addition of species (Figure S5). When *E. coli* was added to 24 h biofilms of the atypical microorganisms, the biomass profile was similar to experiments with synchronized addition of species, regardless of the inoculation proportion between the two species. Also, in dual-species biofilms, the concentration of the initial inoculum did not seem to have a great deal of influence on biomass production over time (Figures 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 CFU 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 (Figures 4, 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. 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 happened in the specific case of the present study remains unclear. It may be, for instance, that the maintenance of atypical species, even at 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* benefits when it 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 and latex rubber). This would allow confirmation of whether these atypical microorganisms are able to degrade certain components of catheters under conditions found in biofilms associated with CAUTIs.

Taken together, these data seem to indicate that species behavior in dual-species biofilm is also dependent

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on the population size and the 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). Indeed, the adhesion of a second colonizer added at low concentration was accelerated. On the other hand, when cellular concentrations reached higher values, the population of atypical species slightly decreased (Figures S2c, d, S7d), which suggests that competition had taken place.

A good example of multispecies biofilm advantages is provided in the work of Lopes et al. (2012). They 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 had the ability to enhance the pathogenicity of this microorganism and, consequently, to influence the outcome of the infection. In addition, other studies also reported the importance of atypical pathogens (eg *Burkholderia cepacia*, *Stenotrophomonas maltophilia* and *A. xylosoxidans*) in clinical outcomes of cystic fibrosis (de Vrankrijker et al. 2010; Waters 2012).

D. tusuruhatensis and *A. xylosoxidans* have been isolated from diverse clinical sources (Lambiase et al. 2011; Preiswerk et al. 2011; Amoureux et al. 2012; Waters 2012; Ciofu et al. 2013), 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 in biofilm architecture and physiology.

Conclusions and future work

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

E. coli presented a greater ability to form a biofilm in conditions mimicking CAUTIs, 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, the coexistence of *E. coli* with the two atypical species within dual-species biofilm structures was proved and pre-colonization with these species seemed to promote adhesion of the pathogen.

The results also suggest that the behavior of species in dual-species biofilm might be dependent on the population size and the space available for growth. Since diversity within the biofilm population usually indicates higher chances of persisting in detrimental conditions,

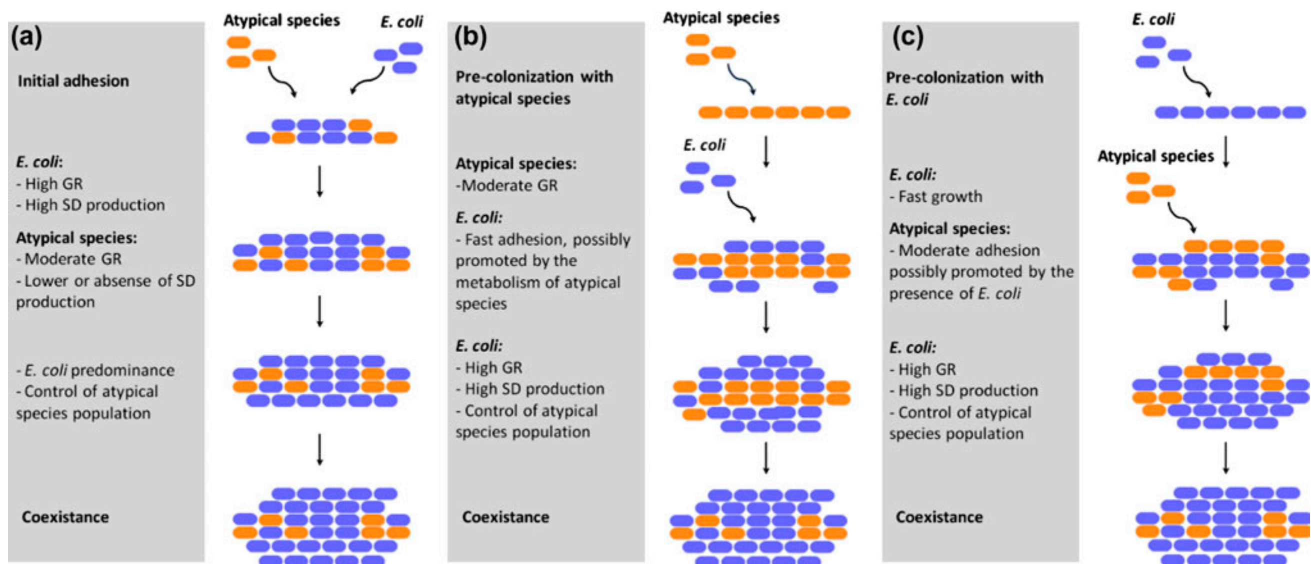

Figure 5. Schematic representation of dual-species biofilm formation showing the main factors involved in the predominance and coexistence of *E. coli* with atypical species. Representation of the interaction between *E. coli* and atypical microorganisms (a) in dual-species biofilm with simultaneous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

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. The high growth rate of *E. coli* in AUM, in association with high levels of siderophore production, helps to explain the ability of this bacterium to outcompete atypical species.

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

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