This article was published in Biofouling, 32(3), 227-241, 2016

http://dx.doi.org/ 10.1080/08927014.2015.1124096

# Impact of *Delftia tsuruhatensis* and *Achromobacter xylosoxidans* on *Escherichia coli* dual-species biofilms treated with antibiotic agents

Andreia S. Azevedo[1,2], Carina Almeida[1,3], Bruno Pereira[1], Luís F. Melo[1], Nuno F. Azevedo[1,*]

[1]Laboratory for Process Engineering, Environment, and Energy and Biotechnology Engineering (LEPABE), Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr Roberto Frias, 4200-465 Porto, Portugal;
[2]Nucleic Acid Center, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, 5230 Odense M, Denmark;
[3]Institute for Biotechnology and Bioengineering (IBB), Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal[c]

Andreia S. Azevedo - deq11016@fe.up.pt

Carina Almeida - carinaalmeida@deb.uminho.pt

Bruno Pereira - bfpereira@ua.pt

Luís F. Melo - lmelo@fe.up.pt

*Corresponding author. E-mail: nazevedo@fe.up.pt; Phone number: +351 22 508 158; Fax number: +351 22 508 1449

*Address correspondence to Nuno F. Azevedo, nazevedo@fe.up.pt

**Abstract**

Recently it was demonstrated that for urinary tract infections species with a lower or unproven pathogenic potential such as *Delftia tsuruhatensis* and *Achromobacter xylosoxidans*, might interact with conventional pathogenic agents as such *Escherichia coli*. In here, single- and dual-species biofilms of these microorganisms were characterized in terms of microbial composition over time, average fitness of *E. coli,* spatial organization and biofilm antimicrobial profile. Results revealed a positive impact of these species on *E. coli* fitness and a greater tolerance to the antibiotic agents. Surprisingly, in dual-species biofilms exposed to antibiotics, *E. coli* was able to dominate the microbial consortia in spite of being the most sensitive strain. This is the first study demonstrating the protective effect of less common species over *E. coli* under adverse conditions imposed by the use of antibiotic agents.

**Keywords:** multispecies biofilms, catheter-associated urinary tract infections, antibiotics, *Escherichia coli*, uncommon species, LNA/2'OMe-FISH.

## Introduction

Healthcare-associated infections include the urinary tract infections (UTIs) (Kline et al. 2012), cystic fibrosis lung disease (Baldan et al. 2014), and device-related infections (Armbruster et al. 2014, Frank et al. 2009, Hola et al. 2010, Stickler 2008) (e.g. urinary catheters). Concerning the catheter-associated urinary tract infections (CAUTIs), *Escherichia coli* is typically one of the most prevailing bacteria (Niveditha et al. 2012, Ronald 2002). Advances in molecular technologies have disclosed that in short-term catheterization, the surface of the urinary catheter is frequently colonized by a single species; while, in the long-term catheterization, a diverse microbial community inhabiting the urinary catheter surface can be observed, with a predominance of gram-negative bacteria (Hola et al. 2010, Hooton et al. 2010, Nicolle 2005). Nonetheless, only a few studies have examined mixed-species structures (eg Azevedo et al. 2014, Cerqueira et al. 2013), and hence, our current knowledge about interspecies dynamic within polymicrobial biofilms, such as microbe-microbe interactions, remains scarce (Elias & Banin 2012).

Two of the species less commonly found on the surface of urinary catheters are *Delftia tsuruhatensis* and *Achromobacter xylosoxidans* (Frank et al. 2009). These species have been shown to be able to coexist with *E. coli* in biofilms, and a pre-colonization of the surface with these species seemed to promote *E. coli* adhesion (Azevedo et al. 2014). While only a limited number of studies have investigated the behavior and role of *E. coli* in catheters-associated polymicrobial biofilms (eg Azevedo et al. 2014, Cerqueira et al. 2013, Spadafino et al. 2014), a previous study has suggested that uncommon bacteria interact synergistically with this pathogen (Azevedo et al. 2014). Similar results were reported for cystic fibrosis associated species, where two other uncommon bacteria, *Inquilinus limosus* and *Dolosigranulum pigrum*, were able to interact synergistically with *Pseudomonas aeruginosa* (Lopes et al. 2012). This type of interaction also resulted into an increased tolerance of the overall consortia to a wide range of antibiotics. Although the pathogenic nature of these uncommon bacteria remains unknown, these studies suggest that some species might cooperate with conventional microorganisms (e.g. *E. coli*, *P. aeruginosa)* to form mixed biofilms in order to protect them from environmentally challenging condition such as antibiotic exposure.

The present study aimed to assess the effect that the uncommon species might have on the fitness and antimicrobial profile of *E. coli* biofilms. *E. coli* and two less common species, *D. tsuruhatensis* and *A. xylosoxidans*, were used to form single- and dual-species biofilms on silicone surfaces. Then, the single- and dual-species biofilms were characterized in terms of microbial

composition over time, average fitness of *E. coli,* spatial organization and biofilm antimicrobial profile.

The interactions, synergetic or antagonistic, among the species within the biofilm have been demonstrated to have a crucial role in the process of biofilm development, architecture and resistance to several antimicrobial agents (Burmolle et al. 2006, Kostaki et al. 2012, Leriche et al. 2003, Simoes et al. 2009). This information might then provide data to model microbial behavior on polymicrobial communities and might also be the base for new personalized treatment strategies (Lopes et al. 2015).

## Materials and methods

### *Culture conditions and preparation of inocula*

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) (Merk, Germany) and grown overnight at 37°C. *E. coli* CECT 434 was originally isolated from a clinical sample in Seattle, Washington, and is often used in quality control testing; *A. xylosoxidans* B3 was isolated from sewage sludge (Reinecke et al. 2000); *D. tsuruhatensis* BM90 was previously isolated from water samples collected at 90 m deep in the Tyrrhenian Sea off the coast of Giglio Island, Grosseto, Italy (Fenice et al. 2007).

For the preparation of the inocula, cells were subcultured (16-18 hours) at 37°C and 150 rpm, in artificial urine medium (AUM). AUM was prepared as previously described (Brooks & Keevil 1997). Cell concentration was assessed by optical density at 620 nm ($O.D._{620\ nm}$), and the inoculum was diluted in AUM in order to obtain a final concentration of $10^5$ CFUs $ml^{-1}$.

### *Single- and dual-species biofilm formation*

Single-species biofilms were formed to study the biofilm-forming ability of each species on silicone material, which is frequently used in urinary catheters (Lawrence & Turner 2005, Stickler 2008). In order to understand the interactions that occur between *E. coli* and the less common microorganisms, 2 species combinations (*E. coli* $10^5$ CFU $ml^{-1}$/ *D. tsuruhatensis* $10^5$ CFU $ml^{-1}$; *E. coli* $10^5$ CFU $ml^{-1}$/ *A. xylosoxidans* $10^5$ CFU $ml^{-1}$) were studied.

Coupons of silicone (Neves & Neves Lda, Portugal) were cut (dimensions of 2 × 2 cm or 1 × 1 cm), cleaned and sterilized according to the procedure described by Azevedo et al. (2006). Each coupon of silicone was placed in the bottom of the wells of the 6-well tissue culture plates (Orange Scientific, Braine-l'Alleud, Belgium).

Cell suspension cultures prepared in AUM at $10^5$ CFUs $ml^{-1}$ were used as an inoculum for biofilm formation. Single- and dual-species biofilms were formed as previously described

(Azevedo et al. 2014). Two independent experiments were performed for each condition. At specific times (2, 4, 6, 24, 48, 96 and 192 h), the biofilm formation was assessed by CFU counts. The spatial organization of dual-species biofilm was also performed using LNA/2'OMe-FISH at 192 h.

***CFU counts for quantification of biofilm cells***

At each time point the silicone coupons with biofilm were washed three times in 10 ml of 0.85% (v/v) sterile saline solution to remove loosely attached cells. After washing, coupons were placed in a new well of the tissue plate containing 9 ml of sterile saline solution 0.85% (v/v); subsequently, the biofilms were sonicated (Sonopuls HD 2070, Bandelin Electronics, Germany) for 10 seconds with 25 % amplitude. The sonication conditions were previously optimized to guarantee that the cells were detached from the silicone coupons, avoiding the bacteria lysis (data not shown). Afterwards, the CFU counts were performed. For this, 100 µl of the disrupted biofilm were serially diluted (1:10) in saline solution, and plated in triplicate on TSA (for the single-species biofilms). The plates were incubated at 37°C for 12–16 h (*E. coli*), 24 h (*D. tsuruhatensis*) and 48 h (*A. xylosoxidans*). For discrimination of the species involved on the dual-species biofilms, different selective agar media were used, as described on Azevedo et al. (2014). MacConkey agar (Liofilchem) was used to assess the *E. coli* CFU counts; and, for assessing the *A. xylosoxidans* and *D. tsuruhatensis* counts the Cetrimide agar (Liofilchem) 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, StLouis, MO, USA]; magnesium sulfate 0.2 g $l^{-1}$ [Merck]; bromothymol blue 0.08 g $l^{-1}$ [Sigma]; agar 13 g $l^{-1}$ [Merck]) were used, respectively. Subsequently, the 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 CFU in biofilms was determined and expressed per unit area of silicone coupon in contact with AUM (Log CFUs $cm^{-2}$). These values were used for the determination of the *E. coli* fitness relative to the less common species ($W_{E.\ coli}$) as previously described by Azevedo et al. (2014).

Briefly, the $W_{E.\ coli}$ was estimated as the ratio of the malthusian parameters (m) of each species (Lenski et al. 1991). This 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 CFU $cm^{-2}$ present in the biofilm at initial time ($t_{initial}$) and final time ($t_{final}$) points. Then, the $W_{E.\ coli}$ was determined as,

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

The value of $W_{E.\ coli}$ indicates the influence of the less common species on the *E. coli* behaviour when co-cultured; a fitness of 1 means that the species are equally fit.

***Antibiotic stock solutions***

Four relevant antibiotics commonly used in the treatment of UTIs and CAUTIs (Dellimore et al. 2013, Hooton et al. 2010, Steinman et al. 2003, Zhanel et al. 2000), with distinct modes of action, were selected, namely: ciprofloxacin (Sigma-Aldrich, Portugal), ampicillin (AppliChem, Germany), gentamicin (AppliChem, Germany), amoxicillin/clavulanic acid (Sigma-Aldrich, Portugal). Stock solution of antibiotics were prepared at 100 000 mg/l. Working solutions were prepared on the day of use at 1024 mg/l, and from these two-fold serial dilutions were made in AUM. The antibiotic concentrations tested ranged from 0.5 to 1024 mg $l^{-1}$.

***Antibiotic susceptibility testing***

The antibiotic susceptibility of single- and dual-species biofilms pre-formed on silicone coupons was evaluated according to Ceri et al. (1999) with slight modifications. Briefly, silicone coupons (1 × 1 cm) were placed on the bottom of the wells of the 24-well tissue culture plates (Orange Scientific, Braine-l'Alleud, Belgium). The biofilm formation was performed as described above. After 48 h, silicone coupons with biofilm were washed three times in 3 ml of 0.85% (v/v) sterile saline and placed in a new well of the tissue culture plate. Then, two-fold serial dilutions of the antibiotic in AUM were applied in the pre-established biofilms and the plates were incubated for 24 h at 37°C, under static conditions. It is important to notice that, at 48 h, the biofilms are mature and the species involved in dual-species biofilms are equally fit.

After the antibiotic exposure, the coupons with biofilms were washed and placed in a new well of the 24-well tissue culture plate containing 1.5 ml of 0.85% (v/v) sterile saline. Subsequently, biofilms were sonicated, as described above, and the suspension of each biofilm was spotted onto TSA plates. The plates were incubated at 37°C for CFU enumeration. These counts allowed to determine the minimum biofilm eradication concentration (MBEC) values, which corresponded to the lower concentration of antibiotic required to eradicate 99% of the sessile bacteria.

***Determination of the species relative composition after antibiotic exposure***

To determine the effect of sub-MBEC concentrations of antibiotics on the species composition in the dual-species biofilms, the CFU enumeration was performed for the concentration close to the MBEC and 8× and 64× lower concentrations. Then, population compositions after and before antibiotic exposure, were compared. A previous study showed a good correlation between

LNA/2'OMe-FISH procedure and CFU counts (Azevedo et al. 2015); therefore, it was considered that the CFU enumeration reflects the population involved in the dual-species biofilms.

***Effect of inoculum size on the species relative composition after antibiotic exposure***

To understand the effect of inoculum size on the species relative composition in antibiotic treated dual-species biofilms, two conditions were tested: i) the antibiotic susceptibility of *E. coli* $10^5$ CFU $ml^{-1}$/ *D. tsuruhatensis* $10^2$ CFU $ml^{-1}$ and *E. coli* $10^5$ CFU $ml^{-1}$/ *A. xylosoxidans* $10^2$ CFU $ml^{-1}$ dual-species biofilms to four antibiotic agents; ii) the susceptibility to ampicillin and amoxicillin/clavulanic acid was tested for the *E. coli* $10^2$ CFU $ml^{-1}$/ *D. tsuruhatensis* $10^5$ CFU $ml^{-1}$ and *E. coli* $10^2$ CFU $ml^{-1}$/ *A. xylosoxidans* $10^5$ CFU $ml^{-1}$ dual-species biofilms. These experiments were performed as described above. The CFU enumeration was also performed for the concentration close to the MBEC and 8× and 64× lower concentrations to determine the species relative composition of each dual-species biofilm.

***Spatial organization of biofilm populations***

In order to assess the biofilm spatial organization and the species distribution, the LNA/2'OMe-FISH procedure in combination with confocal laser scanning microscopy (CLSM) analysis was performed directly on dual-species biofilms formed on silicone coupons at 192 h and on ampicillin treated-biofilms, according to a protocol already developed by Azevedo et al. (2015). Briefly, coupons were washed in 0.85% (v/v) sterile saline; to prevent the detachment of biofilm during hybridization, the biofilms were dried at ~60°C for 15 min and fixed with 100% methanol for 20 min. Afterwards, for the fixation step, the biofilms coupons were immersed in 4% (v/v) paraformaldehyde and 50% (vol/vol) ethanol, for 15 min each at room temperature, and allowed to air dry. Subsequently, a hybridization buffer (0.5 M of urea [VWR BHD Prolabo,], 50 mM Tris-HCl [Fisher Scientific], 0.9 M NaCl [Panreac]; pH 7.5) with 200 nM of the respective probe, were added. The samples were covered with coverslips and incubated in moist chambers at 57°C during 90 min. Next, the coverslips were removed and the coupons were washed in a pre-warmed washing solution (5 mM Tris Base [Fisher Scientific], 15 mM NaCl [Panreac] and 1% Triton X [Panreac]; pH 10) for 30 min at the same temperature of the hybridization step. Finally, the coupons were allowed to air dry before CSLM visualization. The biofilm CSLM images were acquired in a FluoViewFV1000 microscope (Olympus). Biofilm was observed using a 60×water-immersion objective (60×/1.2W). Multichannel simulated fluorescence projection images and vertical cross sections through the biofilm were generated by using the FluoView application Software package (Olympus). *E. coli* cells were identified as green fluorescent bacillus and the uncommon bacteria as bright red fluorescent bacillus.

***Statistical analysis***

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 the SPSS software (SPSS - Statistical Package for the Social Sciences, Chicago, USA). All tests were performed with a confidence level of 95%.

## Results and discussion

Typically, in ecological and clinical environments, biofilm communities are dominated by the species that is better fitted to the environmental conditions (Jacobsen et al. 2008, Lyczak et al. 2002). However, other pathogenic species, or even, species with an unknown pathogenic potential (eg *D. tsuruhatensis* and *A. xyosoxidans*) are also present at a lesser extent (Frank et al. 2009).

Previous reports on *D. tsuruhatensis* and *A. xyosoxidans* species provided relevant information about the type of interactions between these species and *E. coli*, as well as on their impact on biofilm formation and development. While the uncommon species are not directly involved in the pathogenesis of the biofilm, they seemed to help the establishment of the predominant species in the microbial consortium (Azevedo et al. 2014, Lopes et al. 2014, Lopes et al. 2012). As these results were performed in a 96-well plate model, with surfaces that are composed by polystyrene, the first experiments of the present study intended to clarify if this behavior is maintained on silicone surfaces. As such, two consortia composed by the *E. coli* and the less common species (*E. coli*/ *D. tsuruhatensis* and *E. coli*/ *A. xylosoxidans*), formed on silicone surfaces, in AUM, at 37ºC, were studied.

### *Single- and dual-species biofilms growth and spatial organization of the species on silicone material*

First, we assessed the ability of these species to form biofilm on silicone coupons in single- and dual-species biofilms. In single-species biofilms, from 2 h up to 48 h, the CFU counts significantly increased for all species ($P<0.05$). Then, all species stabilized with CFU counts ranging between Log 7.3 CFUs $cm^{-2}$ and Log 7.8 CFUs $cm^{-2}$ (data not shown). These results corroborated the one previously obtained (Azevedo et al. 2014).

To study the influence of the less common species on the $W_{E.\ coli}$, *E. coli* was co-cultured with each of the less common species (Figure 1A). It was clear that at early stages of biofilm formation the $W_{E.\ coli}$ increased significantly in the presence of both less common species ($P<0.05$). For the *E. coli*/ *D. tsuruhatensis* biofilm, this fitness increase was also noticed at 192 h ($P<0.05$). Overall, these results were similar to the results obtained in polystyrene 96-well tissue culture plates (Azevedo et al. 2014), where the less common species and *E. coli* coexisted within the dual-species biofilms at high cell concentrations, with a positive effect on *E. coli* fitness.

The elucidation of the species interactions can be supported by the spatial distribution of the species within the polymicrobial biofilms. It has been shown that particular interactions are associated with specific spatial organizations (Elias & Banin 2012). As such, a multiplex LNA/2'OMe-FISH technique previously validated on biofilm samples (Azevedo et al. 2015) was combined with CLSM to assess the spatial organization of the species in 192 h-dual-species biofilms (Figure 1-B, C). The information allowed to infer the type of interaction that occurs between *E. coli* and the less common species. Images show that the dual-species biofilms were composed by both species mixed together in a typical coaggregation structure. This spatial organization occurs commonly when the species within the biofilm cooperate or interact synergistically (Elias & Banin 2012). Relating this information with data described above, it becomes clear that *E. coli* might benefit from the presence of the *D. tsuruhatensis* and *A. xylosoxidans* or, at least, *E. coli* and these species are not negatively affected by each other's presence, coexisting in the biofilm.

In the synergetic interaction, microorganisms acquire a beneficial phenotype which can result in the development of a stable biofilm, metabolic cooperation, increased resistance to antibiotics and host immune responses (Elias & Banin 2012). Several studies have demonstrated that the polymicrobial consortia are more resistant to antibiotic treatment than the corresponding mono-species biofilms (Al-Bakri et al. 2005, Burmolle et al. 2006, Kara et al. 2006, Leriche et al. 2003). This demonstrated that under challenging conditions imposed by the use of antibacterial agents, the species within the biofilm can cooperate metabolically in order to protect themselves (Elias & Banin 2012, Kara et al. 2006). In fact, the population proportion might be adjusted in order to reach a new balance better suited to the new environmental conditions.

***Antibiotic effects on the relative composition and spatial organization of biofilms formed by E. coli and less common species***

Assuming that the less common species might cooperate with *E. coli* and that this cooperation might have an impact on the antimicrobial profile of the overall microbial consortia, the antibiotic resistance profiles of dual-species biofilms were characterized. Four relevant antibiotics/antibiotic combinations with different modes of action were applied in a 48 h pre-established dual-species biofilms; and, the more prevalent species was determined for the three different antibiotic concentrations below the MBEC.

The antibiotics selected, including ciprofloxacin, gentamicin, ampicillin and amoxicillin/clavulanic acid (from the fluoroquinolone, aminoglycoside and β-lactam drug-class, respectively), are widely used in the treatment of UTIs and CAUTIs (Dellimore et al. 2013, Hooton et al. 2010, Steinman et al. 2003, Zhanel et al. 2000). The MBECs were evaluated for the single- and dual-species biofilms. Results are listed on Table 1.

For the dual-species biofilms (*E. coli* $10^5$ CFU $ml^{-1}$/ *D. tsuruhatensis* $10^5$ CFU $ml^{-1}$; *E. coli* $10^5$ CFU $ml^{-1}$/ *A. xylosoxidans* $10^5$ CFU $ml^{-1}$), it was also expected that higher concentrations of antibiotics were needed to eradicate the consortia than those required to eradicate single-species biofilms; or, at least, an antibiotic concentration equal to the one needed to eradicate the more resistant species which, in this case, were *D. tsuruhatensis* and *A. xylosoxidans*. As expected, in general the MBEC results showed that behavior. An exception was observed for the *E. coli*/ *D. tusuruhatensis* dual-species biofilm where the ciprofloxacin was able to eradicate the biofilm at very low concentration (0.5 mg $l^{-1}$). While individually the single-species biofilms were highly resistance to ciprofloxacin; when combined the resulting mixed biofilm was highly susceptible to the antibiotic. This result reflects how urgent it is to understand the composition and the species interactions in mixed biofilms in order to select a therapy directed to the species involved.

While it was apparent that, in general, the presence of the less common species highly increased the *E. coli* odds of surviving in the presence of antibiotic agents, it was unclear if the exposure to these agents results in a new repositioning of the population balance. To further investigate this, the dual-species biofilm cells were quantified after exposure to antibiotic concentration near or below the MBEC (the CFU $cm^{-2}$ values are present in Supplemental material on Table S2, S3 and S4). Figure 2 shows which species was more prevalent after the introduction of specific antibiotic agents. Before antibiotic exposure, the proportions of the species were similar in both dual-species biofilms with a slight prevalence of *E. coli* (Figure 2-A e B). After antibiotic exposure, results showed that, in general, the relative bacteria composition of the dual-species biofilms was dependent of the antibiotic and concentration applied (Figure 2). For ciprofloxacin, the 3 bacteria presented high MBEC and, thus, the percentages of each population were more balanced. For the other 3 antibiotics, for which MBEC values of the uncommon species were much higher than those obtained for the *E. coli*, a different behavior was observed. It would be expectable that the more resistant species would dominate the microbial consortia. This, in fact, happened for gentamycin-exposed dual-species biofilms, where the percentage of the uncommon species increased with the antibiotic concentration. However, the opposite happened for the ampicillin and amoxicillin/clavulanic acid-exposed biofilms. While the less common species biofilm cells were much more resistant, surprisingly the *E. coli* population dominated the consortia. Both ampicillin and amoxicillin/clavulanic acid belong to the same antibiotic class, the β-lactam class, which might explain the similar results obtained for both antibiotics. The β-lactam antibiotics are able to inhibit the cell wall biosynthesis in the bacterial cell, which ultimately might lead to the cell lysis (Kohanski et al. 2010). Interestingly, biofilms resistance was observed even in the presence of clavulanic acid, which is an inhibitor of the β-lactamase production. These results suggested that a small relative percentage of the *D. tsuruhatensis* and *A. xylosoxidans* was sufficient to introduce some protective changes on the *E. coli* physiology, promoting its resistance and survival against the ampicillin and amoxicillin/clavulanic acid treatment. To determine if this

protective effect exhibited by *D. tsuruhatensis* and *A. xylosoxidans* is maintained in the presence of low initial ratios of the species, the initial inoculum concentration of these species was lowered ($10^2$ CFU $ml^{-1}$) when co-cultured with *E. coli* ($10^5$ CFU $ml^{-1}$).

In general, while the MBEC values decreased due, in part, to a low initial inoculum concentration of *D. tsuruhatensis* and *A. xylosoxidans* (Table 2), the *E. coli* population dominated the consortia after the antibiotic treatment (Figure 3-A, B). The exception was for the ciprofloxacin action on the *E. coli* $10^5$ CFU $ml^{-1}$/ *A. xylosoxidans* $10^2$ CFU $ml^{-1}$ (Figure 3-B). Concerning the gentamycin-treated dual-species biofilms, as previously observed, the results also showed that the *E. coli* population only survived when the antibiotic concentration was below the MBEC of *E. coli* single-species biofilms (Figure 3-A, B).

Next, it was also important to understand whether the *E. coli* population is able to dominate the ampicillin and amoxicillin/clavulanic acid treated biofilms even lowering significantly its initial inoculum concentration ($10^2$ CFU $ml^{-1}$). The results showed that a lower initial inoculum concentration of *E. coli* did not affect the MBEC value to ampicillin and amoxicillin/clavulanic acid of dual-species biofilms (Table S1 in Supplemental material). *E. coli* lost its dominance when co-cultured with *D. tsuruhatensis*, but in general, was able to persist within the consortia (~20% of the total population) (Figure 3-A). When co-cultured with *A. xylosoxidans*, *E. coli* maintained its dominance (Figure 3-B).

The images captured by CLSM revealed that the dual-species biofilms maintain the same structure after treatment with ampicillin (Figure 5). The species are closely associated with a dominance of *E. coli* population (Figure 5-II, VI, VII, VIII). Collectively, these results suggest that the less common species seems to offer a shared resistance to the *E. coli* population, independently of its initial inoculum concentration. In fact, Lee et al. have reported that the role of the species in a consortium is not necessarily related with this abundance; the less abundant species might have a protective effect over the other members involved in the consortia (Lee et al. 2014).

Recently, several studies have demonstrated a new phenomenon of antibiotic resistance based on the "shared resistance" by some members of the microbial consortia (Lee et al. 2014, Perlin et al. 2009, Yurtsev et al. 2013). However, the mechanism underlying this shared resistance is unknown. For the results presented here, three hypotheses are formulated to explain the dominance of the more antibiotic-sensitive species in a multispecies biofilm (Figure 6): 1) the transfer of genetic material from the less common species to *E. coli*; 2) the induction of a different physiological state in the *E. coli* to the antibiotic uptake; 3) the degradation of the antibiotic on the biofilm matrix, through the action of the β-lactamases produced by the uncommon species.

Horizontal gene transfer of antibiotic resistance genes is a way by which some bacteria become resistant to antibiotic agents (Broszat & Grohmann 2014). Recent data have also been provided for the occurrence of this mechanisms in biofilms (Broszat & Grohmann 2014, Ghigo

2001, Hoiby et al. 2010, May et al. 2009, Savage et al. 2013) which may result, in part, from the close cell-to-cell contact occurring in the biofilm (Savage et al. 2013). For instance, the horizontal transfer of specific genes coding for β-lactamases, is not new. May et al. (2009) reported that the resistance to the β-lactam antibiotics is mainly due to the localization of the β-lactamase genes on plasmids, which can spread rapidly among bacteria.

Regarding the induction of a different physiological state, Kara et al. (2006) have suggested this type of interaction for *Streptococcus mutans* and *Veillonella parvula.* When *S. mutans* was co-cultured with *V. parvula*, the latter species induced changes in the gene expression of *S. mutans* allowing its survival under challenging conditions caused by the use of different antibacterial compounds. Another study reported that the sub-inhibitory concentrations of β-lactam antibiotics promotes alterations into the biofilm phenotype, such as a loss of viable bacteria and an increase in biofilm biomass, which can protect and allow the survival of the bacteria exposed to antibiotic agents (Wu et al. 2014).

Regarding the last hypothesis, it was demonstrated that the presence of β-lactamases in the biofilms matrix might inactivate the β-lactam antibiotics (Gould et al. 2008). Lee et al. (2014) suggested that *Pseudomonas protegens* was able to protect all species involved in the microbial consortia (*P. aeruginosa* and *Klebsiella pneumonia*) when exposed to tobramycin, probably due to the ability of the resistant species to produce enzymes that degrade or modify the antibiotics.

In the present study, as previously reported by Lee et al. (2014), there was no selection of the more resistant species (*D. tsuruhatensis* and *A. xylosoxidans*) over the *E. coli* (the less resistant species). The presence of resistant species, even in low concentration, seemed to offer a protection, allowing the survival and dominance of the *E. coli* within microbial consortia under lethal antibiotic concentrations. While the uncommon species (the resistant species) might have their metabolism directed to the secretion of β-lactamases, *E. coli* (the susceptible cells) might gain benefit from the action of β-lactamases secreted. In this situation, *E. coli* does not expend energy in producing enzymes and may redirect that energy to promote its growth and survival without paying the cost. A similar scenario, described by Foster, reports that the resistant cells, through the production of enzymes that break down the antibiotic agents, promote the growth of susceptible cells without cost, conferring a competitive disadvantage for the resistant cells (Foster 2011).

Finally, it is important to note that to ensure the reproducibility of results the use of an established formula of artificial urine was preferred. In fact, human urine varies significantly in terms of pH and compositions according the type of food intake and the health of the individual (Siener et al. 2004). The formula used in this study was reported as a suitable replacement for normal urine and may be used in a wide range of experiments (eg for modelling the growth and attachment of urinary pathogens in the clinical environment) (Almeida et al. 2013, Azevedo et al. 2014, Brooks & Keevil 1997, Cerqueira et al. 2013, Klinth et al. 2012, Raffi et al. 2012). Also,

the use of a dynamic biofilm system might allow to better mimic the urine flow that occurs in the urinary catheter but the experiments were performed on static conditions using the well plates. These platforms offer the possibility of providing a larger amount of data (Duetz 2007, Kumar et al. 2004).

## Conclusions

Interactions between *E. coli* and other less common species in CAUTIs can promote *E. coli* survival under challenging conditions, such as those imposed by the antibiotic agents. A residual concentration of these less common species appears to be sufficient to protect the *E. coli* population. In fact, for certain situations where *E. coli* was more sensitive to the antibiotics than the other microorganisms, it was, nonetheless, able to predominate within the dual-species biofilms. Combining the results obtained in this work, Figure 6 shows a schematic representation of the hypothesis proposed in the present work to explain the predominance of a certain species (*E. coli*, in this case) when a dual-species biofilm is exposed to antibiotics agents or other molecules.

While synergistic interactions between the *E. coli* and the less common species might significantly contribute to the development of well-organized and resistant biofilm structures, it also became clear that some particular species-combination might induce metabolic processes that decrease the resistance mechanism.

In conclusion, this study suggested that there are new aspects about the role of uncommon species that should be investigated such as, how the protection offered by these species contribute to the survival and dominance of sensitive species under lethal antibiotic concentrations. More experiments involving this type of species, and understanding the mechanisms involved in evolution of antibiotic resistance should be taken into consideration. In addition, we suggest that the microbial composition and environmental conditions present in the polymicrobial biofilms should be considered on the development and validation of novel antimicrobial strategies.

## Acknowledgments

This work was financially supported by: Project UID/EQU/00511/2013-LEPABE, by the FCT/MEC with national funds and when applicable co-funded by FEDER in the scope of the P2020 Partnership Agreement; Project NORTE-07-0124-FEDER-000025 - RL2_ Environment&Health, by FEDER funds through Programa Operacional Factores de Competitividade – COMPETE, by the Programa Operacional do Norte (ON2) program and by national funds through FCT - Fundação para a Ciência e a Tecnologia; Project “DNA mimics”

PIC/IC/82815/2007; PhD Fellowship [SFRH/BD/82663/ 2011]; and Postdoctoral Fellowship [SFRH/BPD/74480/2010].

The authors would like to thank to M. Fenice and A. Steinbüchel for kindly providing the *Delftia tsuruhatensis* BM90 and *Achromobacter xylosoxidans* B3 strains, respectively.

## References

Al-Bakri AG, Gilbert P, Allison DG. 2005. Influence of gentamicin and tobramycin on binary biofilm formation by co-cultures of *Burkholderia cepacia* and *Pseudomonas aeruginosa*. J Basic Microbiol. 45:392-396.

Almeida C, Azevedo NF, Bento JC, Cerca N, Ramos H, Vieira MJ, Keevil CW. 2013. Rapid detection of urinary tract infections caused by *Proteus* spp. using PNA-FISH. Eur J Clin Microbiol. 32:781-786.

Armbruster CE, Smith SN, Yep A, Mobley HL. 2014. Increased incidence of urolithiasis and bacteremia during *Proteus mirabilis* and *Providencia stuartii* coinfection due to synergistic induction of urease activity. J Infect Dis. 209:1524-1532.

Azevedo AS, Almeida C, Melo LF, Azevedo NF. 2014. Interaction between atypical microorganisms and *E. coli* in catheter-associated urinary tract biofilms. Biofouling. 30:893-902.

Azevedo AS, Almeida C, Pereira B, Madureira P, Wengel J, Azevedo NF. 2015. Detection and discrimination of biofilm populations using locked nucleic acid/2′-O-methyl-RNA fluorescence *in situ* hybridization (LNA/2′OMe-FISH). Biochemi Eng J. 104:64-73.

Azevedo NF, Pacheco AP, Keevil CW, Vieira MJ. 2006. Adhesion of water stressed *Helicobacter pylori* to abiotic surfaces. J Appl Microbiol. 101:718-724.

Baldan R, Cigana C, Testa F, Bianconi I, De Simone M, Pellin D, Di Serio C, Bragonzi A, Cirillo DM. 2014. Adaptation of *Pseudomonas aeruginosa* in Cystic Fibrosis airways influences virulence of *Staphylococcus aureus in vitro* and murine models of co-infection. PLoS One. 9:e89614.

Brooks T, Keevil CW. 1997. A simple artificial urine for the growth of urinary pathogens. Lett Appl Microbiol. 24:203-206.

Broszat M, Grohmann E. 2014. Antibiofilm Agents. Springer Berlin Heidelberg. Chpter 5, Horizontal Gene Transfer in Planktonic and Biofilm Modes; p. 67-95.

Burmolle M, Webb JS, Rao D, Hansen LH, Sorensen SJ, Kjelleberg S. 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Appl Environ Microbiol. 72:3916-3923.

Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. 1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol. 37:1771-1776.

Cerqueira L, Oliveira JA, Nicolau A, Azevedo NF, Vieira MJ. 2013. Biofilm formation with mixed cultures of *Pseudomonas aeruginosa*/*Escherichia coli* on silicone using artificial urine to mimic urinary catheters. Biofouling. 29:829-840.

Dellimore KH, Helyer AR, Franklin SE. 2013. A scoping review of important urinary catheter induced complications. J Mater Sci Mater Med. 24:1825-1835.

Duetz WA. 2007. Microtiter plates as mini-bioreactors: miniaturization of fermentation methods. Trends Microbiol. 15:469-475.

Elias S, Banin E. 2012. Multi-species biofilms: living with friendly neighbors. FEMS Microbiol Rev. 36:990-1004.

491 Fenice M, Gallo A, Juarez-Jimenez B, Gonzalez-Lopez J. 2007. Screening for extracellular
492 enzyme activities by bacteria isolated from samples collected in the Tyrrhenian Sea. Ann
493 Microbiol. 57:93-99.
494 Foster KR. 2011. The Secret Social Lives of Microorganisms. Microbe. 6:183-186.
495 Frank DN, Wilson SS, St Amand AL, Pace NR. 2009. Culture-independent microbiological
496 analysis of foley urinary catheter biofilms. PLoS One. 4:e7811.
497 Ghigo JM. 2001. Natural conjugative plasmids induce bacterial biofilm development. Nature.
498 412:442-445.
499 Gould I, van der Meer J, Ciofu O, Høiby N. 2008. Antibiotic Policies: Fighting Resistance.
500 Springer US. Cystic Fibrosis-Coping with Resistance; p. 149-174.
501 HØiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. 2010. Antibiotic resistance of bacterial
502 biofilms. Int J Antimicrob Agents. 35:322-332.
503 Hola V, Ruzicka F, Horka M. 2010. Microbial diversity in biofilm infections of the urinary tract
504 with the use of sonication techniques. FEMS Immunol MedMicrobiol. 59:525-528.
505 Hooton TM, Bradley SF, Cardenas DD, Colgan R, Geerlings SE, Rice JC, Saint S, Schaeffer AJ,
506 Tambayh PA, Tenke P, et al. 2010. Diagnosis, prevention, and treatment of catheter-associated
507 urinary tract infection in adults: 2009 International Clinical Practice Guidelines from the
508 Infectious Diseases Society of America. Clin Infect Dis. 50:625-663.
509 Jacobsen SM, Stickler DJ, Mobley HL, Shirtliff ME. 2008. Complicated catheter-associated
510 urinary tract infections due to *Escherichia coli* and *Proteus mirabilis*. Clin Microbiol Rev. 21:26-
511 59.
512 Kara D, Luppens SB, Cate JM. 2006. Differences between single- and dual-species biofilms of
513 *Streptococcus mutans* and *Veillonella parvula* in growth, acidogenicity and susceptibility to
514 chlorhexidine. Eur J Oral Sci. 114:58-63.
515 Kline KA, Schwartz DJ, Gilbert NM, Hultgren SJ, Lewis AL. 2012. Immune modulation by group
516 B Streptococcus influences host susceptibility to urinary tract infection by uropathogenic
517 *Escherichia coli*. Infect Immun. 80:4186-4194.
518 Klinth JE, Castelain M, Uhlin BE, Axner O. 2012. The influence of pH on the specific adhesion
519 of P piliated *Escherichia coli*. PLoS One. 7:e38548.
520 Kohanski MA, Dwyer DJ, Collins JJ. 2010. How antibiotics kill bacteria: from targets to
521 networks. Nat Rev Microbiol. 8:423-435.
522 Kostaki M, Chorianopoulos N, Braxou E, Nychas GJ, Giaouris E. 2012. Differential biofilm
523 formation and chemical disinfection resistance of sessile cells of listeria monocytogenes strains
524 under monospecies and dual-species (with *Salmonella enterica*) conditions. Appl Environ
525 Microbiol. 78:2586-2595.
526 Kumar S, Wittmann C, Heinzle E. 2004. Review: Minibioreactors. Biotechnol Lett. 26:1-10.
527 Lawrence EL, Turner IG. 2005. Materials for urinary catheters: a review of their history and
528 development in the UK. Med Eng Phys. 27:443-453.
529 Lee KW, Periasamy S, Mukherjee M, Xie C, Kjelleberg S, Rice SA. 2014. Biofilm development
530 and enhanced stress resistance of a model, mixed-species community biofilm. ISME J. 8:894-
531 907.
532 Lenski RE, Rose MR, Simpson SC, Tadler SC. 1991. Long-Term Experimental Evolution in
533 *Escherichia coli*. I. Adaptation and Divergence During 2,000 Generations. Am Nat. 138:1315-
534 1341.
535 Leriche V, Briandet R, Carpentier B. 2003. Ecology of mixed biofilms subjected daily to a
536 chlorinated alkaline solution: spatial distribution of bacterial species suggests a protective effect
537 of one species to another. Environ Microbiol. 5:64-71.

538 Lopes SP, Azevedo NF, Pereira MO. 2014. Emergent bacteria in cystic fibrosis: *in vitro* biofilm
539 formation and resilience under variable oxygen conditions. Biomed Res Int. 2014:678301.
540 Lopes SP, Azevedo NF, Pereira MO. 2015. Microbiome in cystic fibrosis: Shaping polymicrobial
541 interactions for advances in antibiotic therapy. Crit Rev Microbiol. 41:353-365.
542 Lopes SP, Ceri H, Azevedo NF, Pereira MO. 2012. Antibiotic resistance of mixed biofilms in
543 cystic fibrosis: impact of emerging microorganisms on treatment of infection. Int J Antimicrob
544 Agents. 40:260-263.
545 Lyczak JB, Cannon CL, Pier GB. 2002. Lung infections associated with cystic fibrosis. Clin
546 Microbiol Rev. 15:194-222.
547 May T, Ito A, Okabe S. 2009. Induction of multidrug resistance mechanism in *Escherichia coli*
548 biofilms by interplay between tetracycline and ampicillin resistance genes. Antimicrob Agents
549 Chemother. 53:4628-4639.
550 Nicolle LE. 2005. Catheter-related urinary tract infection. Drugs Aging. 22:627-639.
551 Niveditha S, Pramodhini S, Umadevi S, Kumar S, Stephen S. 2012. The isolation and the biofilm
552 formation of uropathogens in the patients with catheter associated urinary tract infections (UTIs).
553 J Clin Diagn Res. 6:1478-1482.
554 Perlin MH, Clark DR, McKenzie C, Patel H, Jackson N, Kormanik C, Powell C, Bajorek A,
555 Myers DA, Dugatkin LA, et al. 2009. Protection of Salmonella by ampicillin-resistant
556 *Escherichia coli* in the presence of otherwise lethal drug concentrations. Proc Biol Sci. 276:3759-
557 3768.
558 Raffi HS, Bates JM, Flournoy DJ, Kumar S. 2012. Tamm-Horsfall protein facilitates catheter
559 associated urinary tract infection. BMC Res Notes. 5:532.
560 Reinecke F, Groth T, Heise KP, Joentgen W, Muller N, Steinbuchel A. 2000. Isolation and
561 characterization of an *Achromobacter xylosoxidans* strain B3 and other bacteria capable to
562 degrade the synthetic chelating agent iminodisuccinate. FEMS Microbiol Lett. 188:41-46.
563 Ronald A. 2002. The etiology of urinary tract infection: traditional and emerging pathogens. Am
564 J Med. 113:14S-19S.
565 Savage VJ, Chopra I, O'Neill AJ. 2013. *Staphylococcus aureus* biofilms promote horizontal
566 transfer of antibiotic resistance. Antimicrob Agents Chemother. 57:1968-1970.
567 Siener R, Jahnen A, Hesse A. 2004. Influence of a mineral water rich in calcium, magnesium and
568 bicarbonate on urine composition and the risk of calcium oxalate crystallization. Eur J Clin Nutr.
569 58:270-276.
570 Simoes M, Simoes LC, Vieira MJ. 2009. Species association increases biofilm resistance to
571 chemical and mechanical treatments. Water Res. 43:229-237.
572 Spadafino JT, Cohen B, Liu J, Larson E. 2014. Temporal trends and risk factors for extended-
573 spectrum beta-lactamase-producing *Escherichia coli* in adults with catheter-associated urinary
574 tract infections. Antimicrob Resist Infect Control. 3:39.
575 Steinman MA, Gonzales R, Linder JA, Landefeld CS. 2003. Changing use of antibiotics in
576 community-based outpatient practice, 1991-1999. Ann Intern Med. 138:525-533.
577 Stickler DJ. 2008. Bacterial biofilms in patients with indwelling urinary catheters. Nat Clin Pract
578 Urol. 5:598-608.
579 West SA, Griffin AS, Gardner A, Diggle SP. 2006. Social evolution theory for microorganisms.
580 Nat Rev Micro. 4:597-607.
581 Wu S, Li X, Gunawardana M, Maguire K, Guerrero-Given D, Schaudinn C, Wang C, Baum MM,
582 Webster P. 2014. Beta-lactam antibiotics stimulate biofilm formation in non-typeable
583 haemophilus influenzae by up-regulating carbohydrate metabolism. PLoS One. 9:e99204.
584 Yurtsev EA, Chao HX, Datta MS, Artemova T, Gore J. 2013. Bacterial cheating drives the
585 population dynamics of cooperative antibiotic resistance plasmids. Mol Syst Biol. 9:683.

Zhanel GG, Karlowsky JA, Harding GK, Carrie A, Mazzulli T, Low DE, Hoban DJ. 2000. A Canadian national surveillance study of urinary tract isolates from outpatients: comparison of the activities of trimethoprim-sulfamethoxazole, ampicillin, mecillinam, nitrofurantoin, and ciprofloxacin. The Canadian Urinary Isolate Study Group. Antimicrob Agents Chemother. 44:1089-1092.

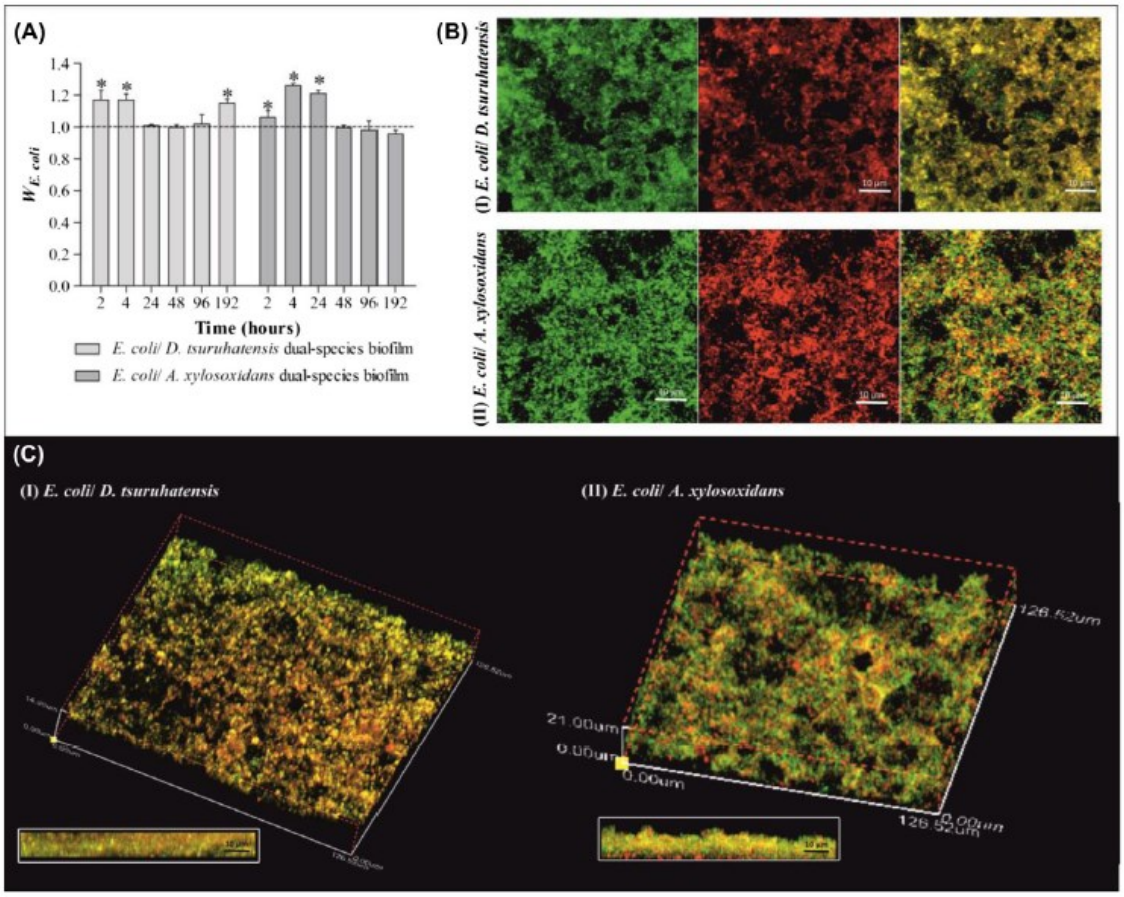


Figure 1. Single and dual-species biofilm growth on silicone material.
Notes: The three species were individually cultured or co-cultured at 37°C, on silicone coupons, under static conditions. Two independent experiments were performed for each condition. Error bars represent the SD. (A) Representation of the relative fitness of *E. coli* when co-cultured with the uncommon species (*D. tsuruhatensis* and *A. xylosoxidans*). The dashed line represents a relative fitness of 1, which means that the species are equally fit. The asterisk (*) placed over the bars indicates a statistically significant difference between the relative fitness of *E. coli* in dual-species biofilms and 1 ($p < 0.05$). (B) CLSM images of (I) *E. coli* $10_5$ CFU $ml_{-1}$/*D. tsuruhatensis* $10_5$ CFU $ml_{-1}$ and (II) *E. coli* $10_5$ CFU $ml_{-1}$/*A. xylosoxidans* $10_5$ CFU $ml_{-1}$, distinguishing each bacterium in two different fluorescence channels and the superposition of the two fields. (C) CLSM showing the spatial organization of the biofilm of (I) *E. coli* $10_5$ CFU $ml_{-1}$/*D. tsuruhatensis* $10_5$ CFU $ml_{-1}$ and (II) *E. coli* $10_5$ CFU $ml_{-1}$/*A. xylosoxidans* $10_5$ CFU $ml_{-1}$ 192 h dual-species biofilms. The bottom images represent the transverse planes.

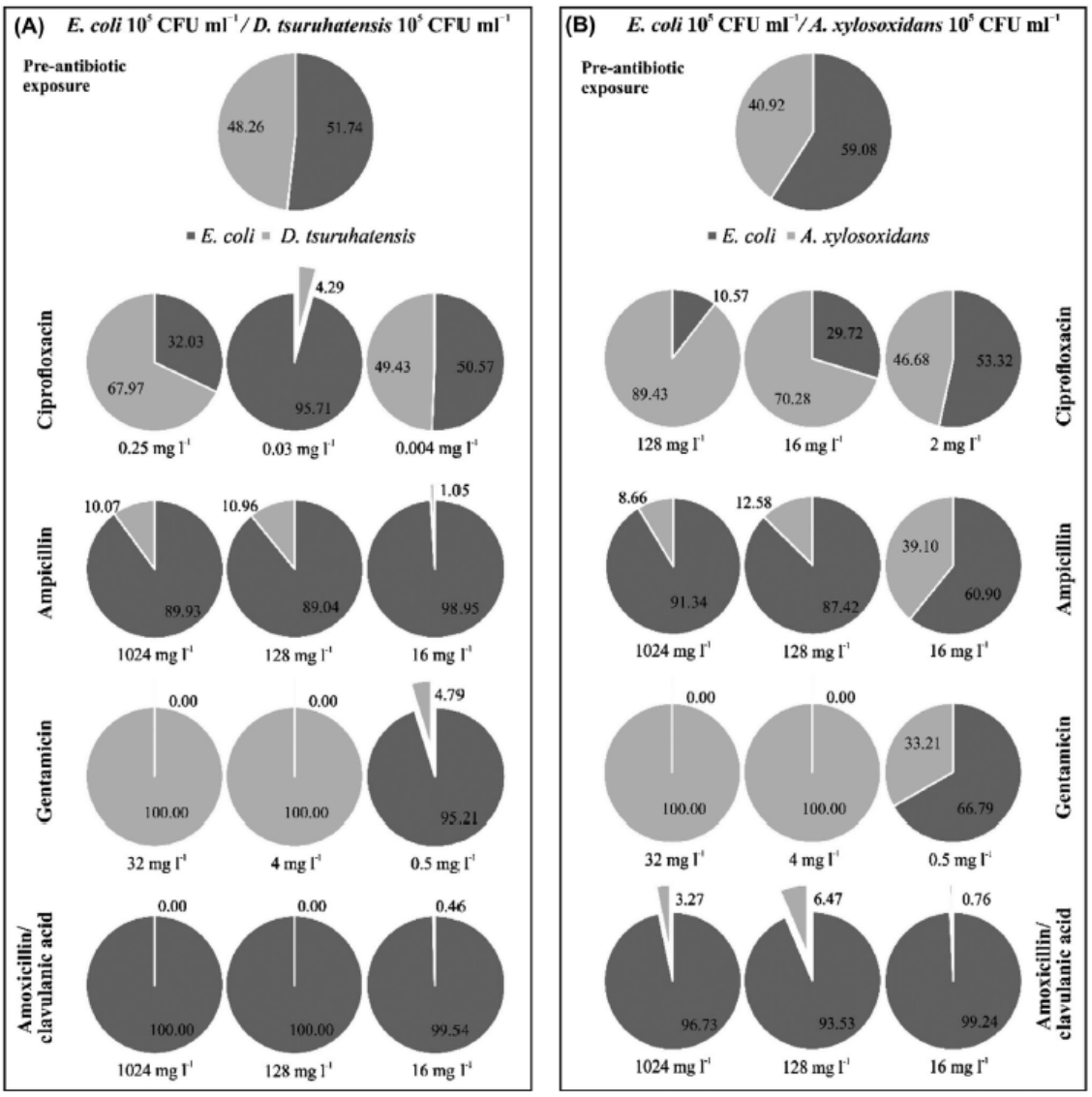


Figure 2. Relative bacterial composition of the dual-species biofilms after antibiotic exposure. Notes: For each antibiotic, the *E. coli*/*D. tsuruhatensis* (A) and *E. coli*/*A. xylosoxidans* (B) 48 h dual-species biofilms were exposed to three different concentrations below the MBEC; then, the CFU counts were determined after exposure for 24 h. An initial inoculum concentration of $10_5$ CFU $ml_{-1}$ was used for these experiments. Two independent experiments were performed for each condition.

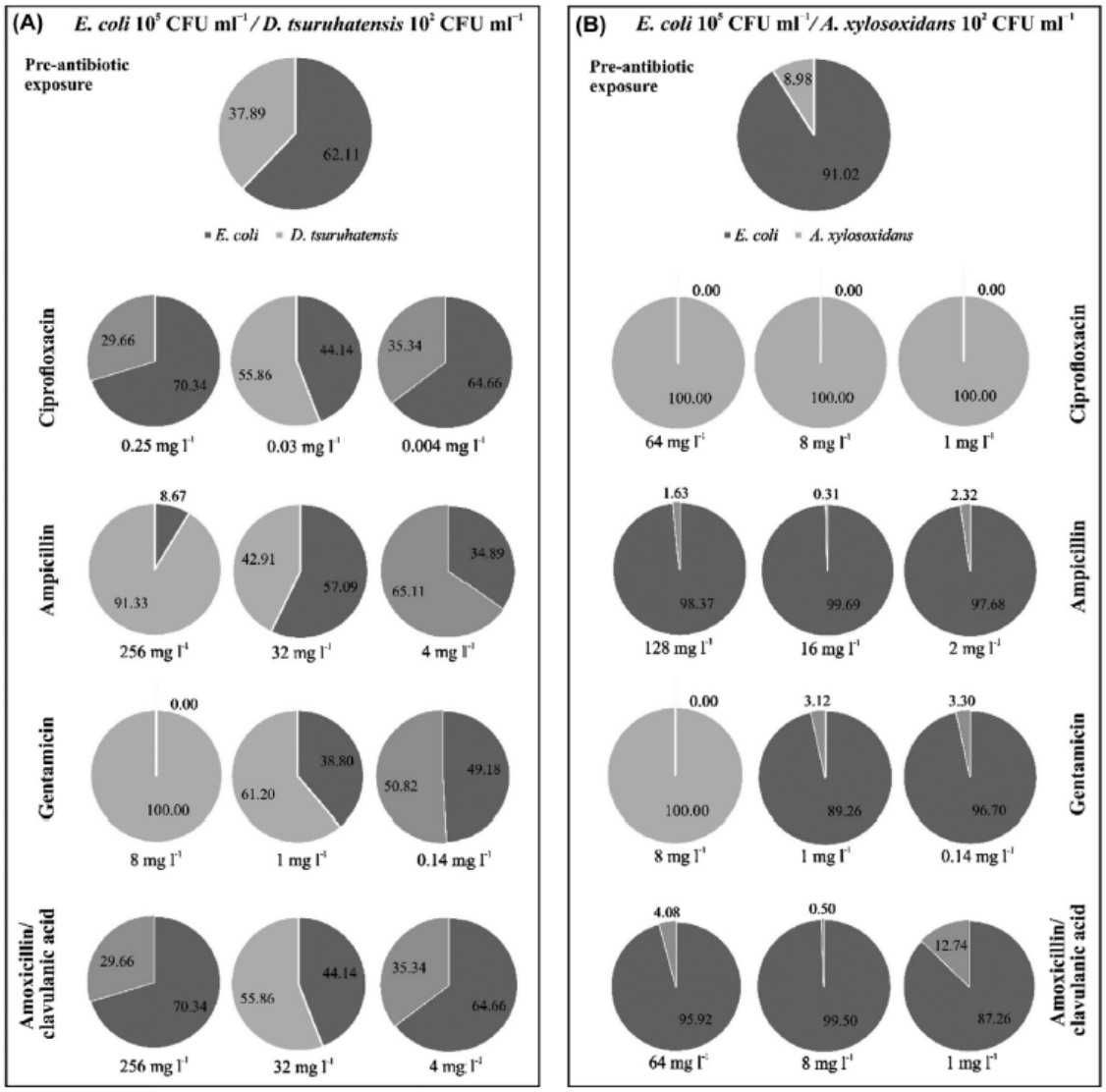


Figure 3. Effect of a lower *D. tsuruhatensis* or *A. xylosoxidans* initial inoculum concentration ($10_2$ CFU $ml_{-1}$) on the *E. coli* population after antibiotic exposure. Notes: For each antibiotic, the *E. coli*/*D. tsuruhatensis* (A) and *E. coli*/*A. xyosoxidans* (B) 48 h dual-species biofilms were exposed to three different concentrations below the MBEC, then, the CFU counts were determined after exposure for 24 h. An *E. coli* initial inoculum concentration of $10_5$ CFU $ml_{-1}$ was used for these experiments. Two independent experiments were performed for each condition.

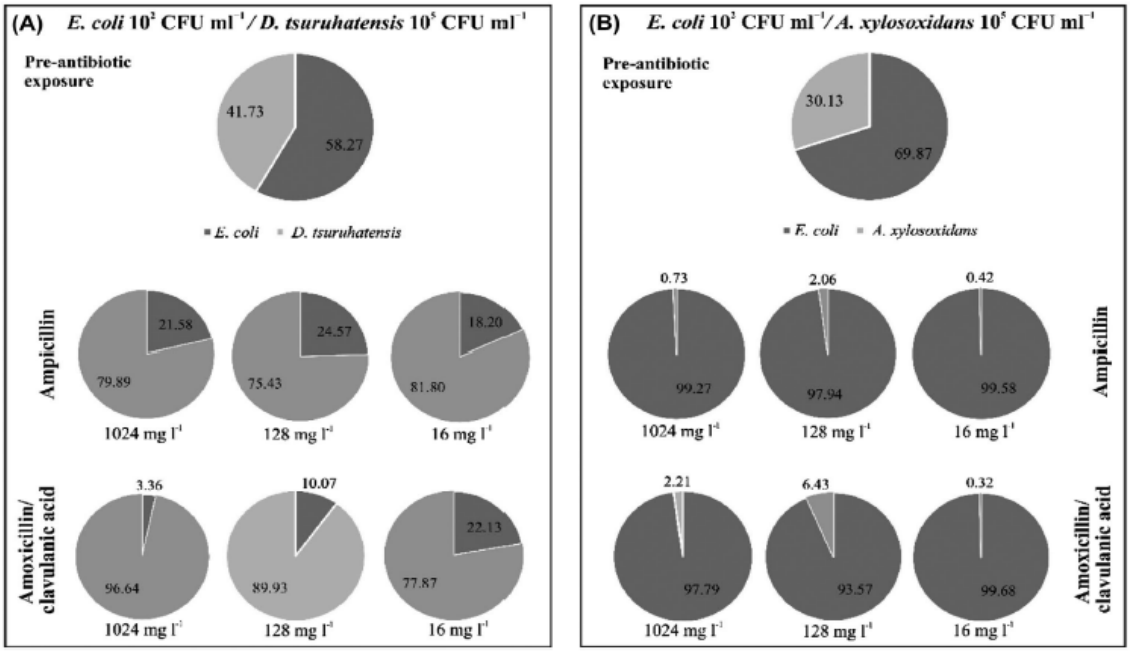


Figure 4. Effect of a lower *E. coli* initial inoculum concentration ($10_2$ CFU $ml_{-1}$) on the relative bacterial composition of the dual-species biofilms after exposure to ampicillin and amoxicillin/clavulanic acid. Notes: For each antibiotic, the *E. coli*/*D. tsuruhatensis* (A) and *E. coli*/*A. xyosoxidans* (B) 48 h dual-species biofilms were exposed to three different concentrations below the MBEC, then, the CFU counts were determined after exposure for 24 h. An initial inoculum concentration of $10_5$ CFU $ml_{-1}$ for *D. tsuruhatensis* and *A. xylosoxidans* was used for these experiments. Two independent experiments were performed for each condition.

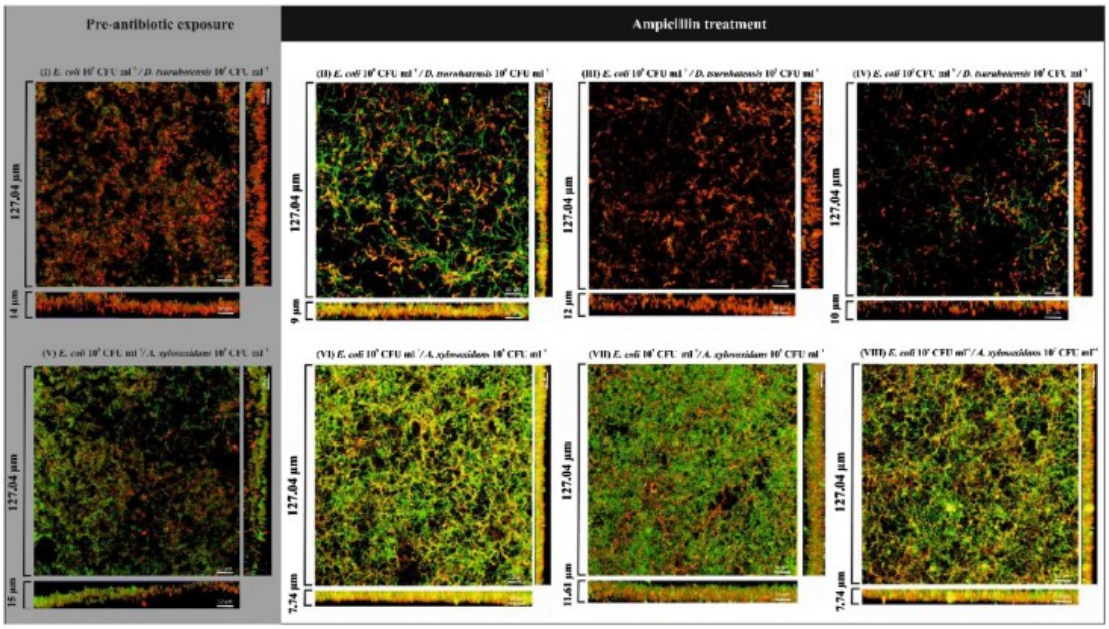


Figure 5. Spatial localization and structure of dual-species biofilms exposed to ampicillin. Notes: CLSM images showing the spatial organization of the biofilm of (I) *E. coli* $10_5$ CFU $ml_{-1}$/*D. tsuruhatensis* $10_5$ CFU $ml_{-1}$ before the ampicillin exposure; and (II) *E. coli* $10_5$ CFU $ml_{-1}$/ *D. tsuruhatensis* $10_5$ CFU $ml_{-1}$, (III) *E. coli* $10_5$ CFU $ml_{-1}$/*D. tsuruhatensis* $10_2$ CFU $ml_{-1}$, and (IV) *E. coli* $10_2$ CFU $ml_{-1}$/*D. tsuruhatensis* $10_5$ CFU $ml_{-1}$ after ampicillin treatment; (V) *E. coli* $10_5$ CFU $ml_{-1}$/*A. xylosoxidans* $10_5$ CFU $ml_{-1}$ before the ampicillin exposure; and (VI) *E. coli* $10_5$ CFU $ml_{-1}$/*A. xylosoxidans* $10_5$ CFU $ml_{-1}$, (VII) *E. coli* $10_5$ CFU $ml_{-1}$/*A. xylosoxidans* $10_2$ CFU $ml_{-1}$, and (VIII) *E. coli* $10_2$ CFU $ml_{-1}$/*A. xylosoxidans* $10_5$ CFU $ml_{-1}$ after ampicillin treatment. The bottom and side images of each panel represent the transverse planes.

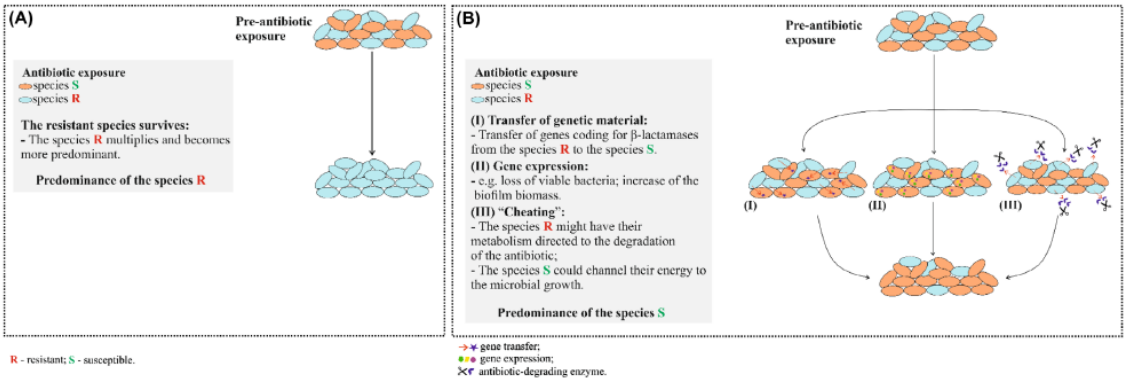


Figure 6. Schematic representation of the hypothesis proposed in the present work to explain the predominance of a certain species when a dual-species biofilm is exposed to antibiotics.
Notes: After antibiotic exposure, (A) typically the species more resistant survives and dominates the microbial consortia; (B) however, the opposite can occur, and, the more susceptible species resists and dominates. Some hypothesis to explain that observation include: (1) transfer of genetic material from the resistant species to the susceptible species; (2) induction of a different physiological state in the susceptible species due to antibiotic uptake; (3) degradation of the antibiotic in the biofilm matrix, through the action of the enzymes produced by the resistant species.

Table 1. MBEC values for *E. coli*, *D. tsuruhatensis* and *A. xylosoxidans* single- and dual-species biofilms, exposed to four antibiotics.

| | Antibiotic (mg $l^{-1}$) | | | |
|---|---|---|---|---|
| | Ciprofloxacin | Ampicillin | Gentamicin | Amoxicillin/clavulanic acid* |
| | MBEC | MBEC | MBEC | MBEC |
| *E. coli* | 256 | 128 | 2 | 64/9.15 |
| *D. tsuruhatensis* | 256 | >1,024 | 256 | >1,024/146.29 |
| *A. xylosoxidans* | 256 | >1,024 | 32 | >1,024/146.29 |
| *E. coli*/*D. tsuruhatensis* | 0.50 | >1,024 | 64 | >1,024/146.29 |
| *E. coli*/*A. xylosoxidans* | 256 | >1,024 | 64 | >1,024/146.29 |

*Ratio 1/7 used in clinical treatments; MBEC, minimum biofilm eradication concentration.
Note: An initial inoculum concentration of $10^5$ CFU $ml^{-1}$ was used for these experiments.

Table 2. Effect of a low *D. tsuruhatensis* or *A. xylosoxidans* initial inoculum concentration ($10_2$ CFU $ml_{-1}$) on the *in vitro* susceptibility of the dual-species biofilms to four antibiotics.

| | Antibiotic (mg $l^{-1}$) | | | |
|---|---|---|---|---|
| | Ciprofloxacin | Ampicillin | Gentamicin | Amoxicillin/clavulanic acid* |
| | MBEC | MBEC | MBEC | MBEC |
| *E. coli* $10^5$ CFU $ml^{-1}$/*D. tsuruhatensis* $10^2$ CFU $ml^{-1}$ | 0.5 | 512 | 16 | 512 |
| *E. coli* $10^5$ CFU $ml^{-1}$/*A. xylosoxidans* $10^2$ CFU $ml^{-1}$ | 128 | 256 | 16 | 128 |

*Ratio 1/7 used in clinical treatments; MBEC, minimum biofilm eradication concentration.