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

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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 such as *Escherichia coli*. Here, single- and dual-species biofilms of these microorganisms were characterized in terms of microbial composition over time, the average fitness of *E. coli*, the spatial organization and the biofilm antimicrobial profile. The results revealed a positive impact of these species on the fitness of *E. coli* and a greater tolerance to the antibiotic agents. 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.

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Introduction

Healthcare-associated infections include urinary tract infections (UTIs) (Kline et al. 2012), cystic fibrosis lung disease (Baldan et al. 2014), and device-related infections (Stickler 2008; Frank et al. 2009; Hola et al. 2010; Armbruster et al. 2014), eg urinary catheters. *Escherichia coli* is typically one of the predominant bacteria in catheter-associated urinary tract infections (CAUTIs) (Ronald 2002; Niveditha et al. 2012). 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 long-term catheterization a diverse microbial community inhabiting the urinary catheter surface can be observed, with a predominance of Gram-negative bacteria (Nicolle 2005; Hola et al. 2010; Hooton et al. 2010). Nonetheless, only a few studies have examined mixed-species structures (eg Cerqueira et al. 2013; Azevedo et al. 2014), and hence current knowledge on the 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 Cerqueira et al. 2013; Azevedo et al. 2014; 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 in 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 (eg *E. coli*, *P. aeruginosa*) to form mixed biofilms in order to protect them from environmentally challenging conditions such as antibiotic exposure.

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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. Single- and dual-species biofilms were characterized in terms of the microbial composition over time, the average fitness of *E. coli*, the spatial organization and the 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 (Leriche et al. 2003; Burmolle et al. 2006; Simoes et al. 2009; Kostaki et al. 2012). This information might 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) (Merck, Darmstadt, Germany) and grown overnight at 37°C. *E. coli* CECT 434 was originally isolated from a clinical sample in Seattle, WA, USA, 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 a depth of 90 m 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 h) 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 ($OD_{620\ nm}$), and the inoculum was diluted in AUM in order to obtain a final concentration of 10^5 CFU 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, two 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, Porto,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 was placed in the bottom of the wells of six-well tissue culture plates (Orange Scientific, Braine-l'Alleud, Belgium).

Cell suspension cultures prepared in AUM at 10^5 CFU 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), biofilm formation was assessed by CFU counts. The spatial organization of dual-species biofilm was also performed using locked nucleic acid/2′-O-methyl-RNA fluorescence in situ hybridization (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, Berlin, Germany) for 10 s with an amplitude of 25%. The sonication conditions were previously optimized to guarantee that the cells were detached from the silicone coupons, avoiding bacterial lysis (data not shown). Afterwards, 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 in the dual-species biofilms, different selective agar media were used, as described by Azevedo et al. (2014). MacConkey agar (Liofilchem, Roseto degli Abruzzi, Italia) was used to assess the *E. coli* CFU counts, and, for assessing the *A. xylosoxidans* and *D. tsuruhatensis* counts, 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, 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]) 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 CFUs in biofilms was determined and expressed per unit area of silicone coupon in contact with AUM (log CFU cm^{-2}). These values were used for the determination of

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 the 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 behavior of *E. coli* 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 (Zhanel et al. 2000; Steinman et al. 2003; Hooton et al. 2010; Dellimore et al. 2013), with distinct modes of action, were selected, namely: ciprofloxacin (Sigma-Aldrich, Sintra, Portugal), ampicillin (AppliChem, Darmstadt, Germany), gentamicin (AppliChem), and amoxicillin/clavulanic acid (Sigma-Aldrich). Stock solution of the antibiotics were prepared at 100 g l^{-1}. Working solutions were prepared on the day of use at 1,024 mg l^{-1}, and from these twofold serial dilutions were made in AUM. The antibiotic concentrations tested ranged from 0.5 to 1,024 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). Biofilm formation was performed as described above. After 48 h, the 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, twofold 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 note 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, the 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 enabled determination of 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 the LNA/2′OMe-FISH procedure and CFU counts (Azevedo et al. 2015), therefore, it was considered that 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 relative species composition in antibiotic treated dual-species biofilms, two tests were performed: (1) the antibiotic susceptibility to four antibiotic agents was tested in *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; and (2) 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 relative species composition of each dual-species biofilm.

Spatial organization of biofilm populations

In order to assess the spatial organization of the biofilms 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 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 immersed in 100% methanol for 20 min. Afterwards, for

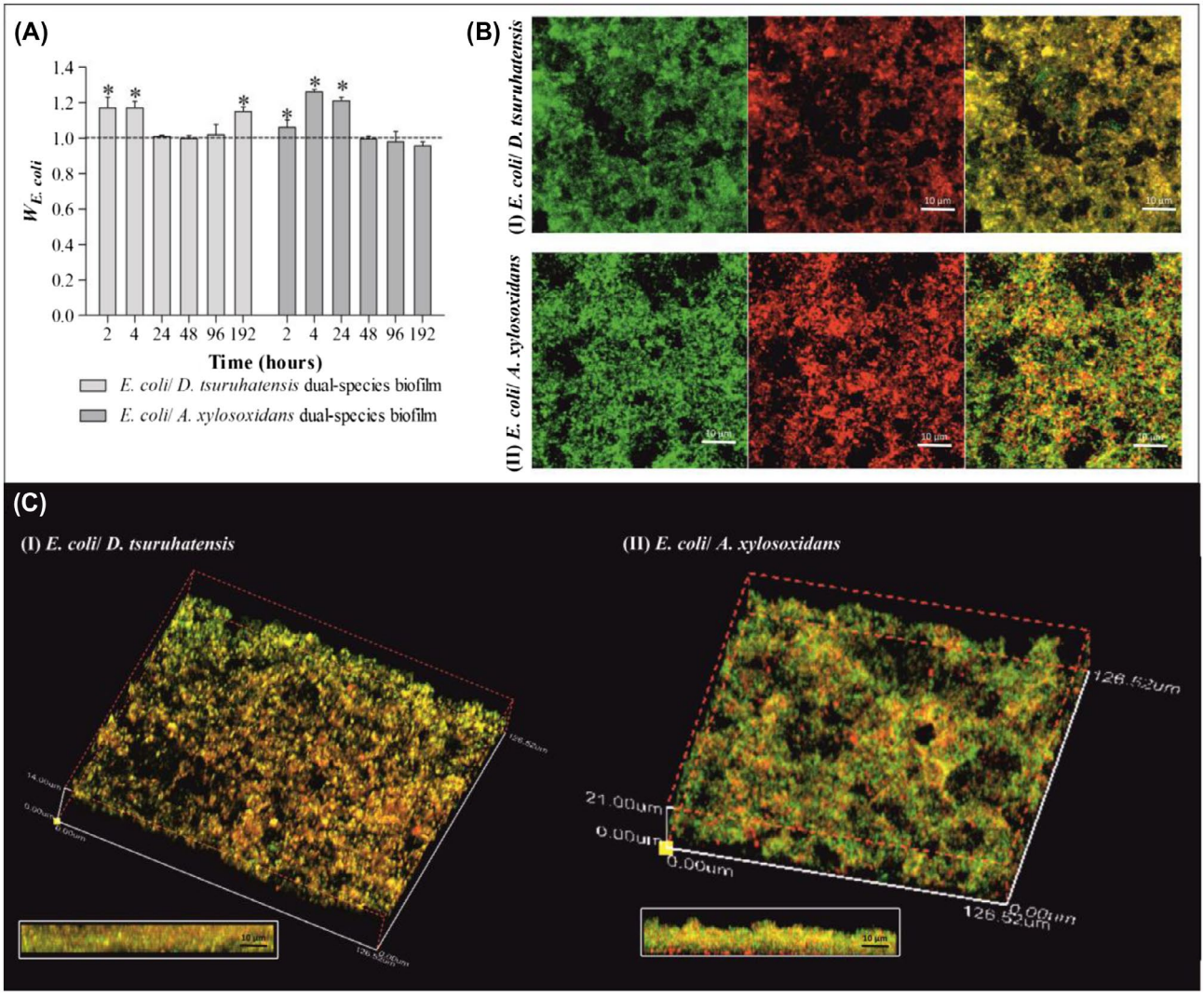

Figure 1. Single and dual-species biofilm growth on silicone material.
Notes: The three species were individually cultured or co-cultured at 37°C, on silicone coupons, under static conditions. Two independent experiments were performed for each condition. Error bars represent the SD. (A) Representation 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.

the fixation step, the biofilm coupons were immersed in 4% (v/v) paraformaldehyde and 50% (v/v) ethanol, for 15 min each at room temperature, and allowed to air dry. Subsequently, a hybridization buffer (0.5 M of urea [VWR BDH Prolabo, Carnaxide, Portugal], 50 mM Tris-HCl [Fisher Scientific, Lisbon, Portugal], 0.9 M NaCl [Panreac, Barcelona, Spain]; 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 for 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 as the hybridization step. Finally, the coupons were allowed to air dry before CSLM visualization. The CSLM images of the biofilms were acquired in a FluoView FV1000 microscope (Olympus, Hamburg, Germany). Biofilm was observed using a 60 × water-immersion objective (60 × /1.2 W). Multichannel simulated fluorescence projection images and vertical cross sections through the biofilm were generated by using the FluoView application software

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.

package (Olympus). *E. coli* cells were identified as green fluorescent bacilli and the uncommon bacteria as bright red fluorescent bacilli.

Statistical analysis

The 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 (Statistical Package for the Social Sciences, Chicago, IL, 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 (Lyczak et al. 2002; Jacobsen et al. 2008). However, other pathogenic species, or even species with an unknown pathogenic potential (eg *D. tsuruhatensis* and *A. xyosoxidans*) are also present to 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 (Lopes et al. 2012, 2014; Azevedo et al. 2014). As these experiments were performed in a 96-well plate model, with polystyrene surfaces, the first experiments in the present study were intended to clarify whether this behavior is maintained on silicone surfaces. As such, two consortia composed of *E. coli* and the less common species (*E. coli*/*D. tsuruhatensis* and *E. coli*/*A. xylosoxidans*), formed on silicone surfaces in AUM at 37°C, were studied.

Single- and dual-species biofilm growth and the spatial organization of the species on silicone material

First, the ability of these species to form biofilm on silicone coupons in single- and dual-species biofilms was assessed. In single-species biofilms, from 2 h 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 CFU cm^{-2} and log 7.8 CFU cm^{-2} (data not shown). These results corroborated those 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 of the 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 those 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 1B and C). The information allowed the type of interaction that occurs between *E. coli* and the less common species to be inferred. Images show that the dual-species biofilms were composed of both species mixed together in a typical coaggregation structure. This spatial organization occurs

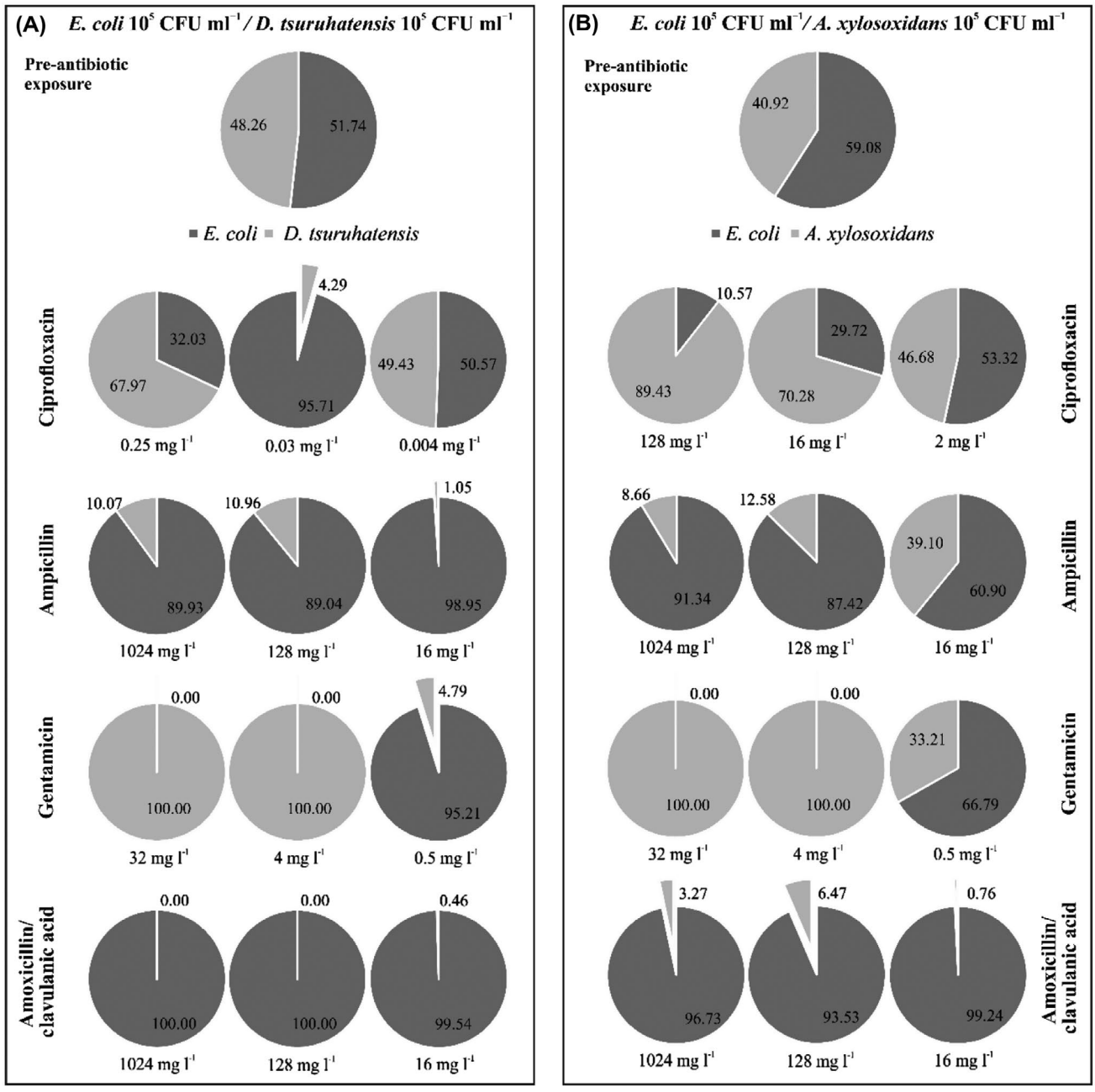

Figure 2. Relative bacterial composition of the dual-species biofilms after antibiotic exposure.
Notes: For each antibiotic, the *E. coli*/*D. tsuruhatensis* (A) and *E. coli*/*A. xylosoxidans* (B) 48 h dual-species biofilms were exposed to three different concentrations below the MBEC; then, the CFU 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.

commonly when the species within a biofilm cooperate or interact synergistically (Elias & Banin 2012). Relating this information to the data described above, it becomes clear that *E. coli* might benefit from the presence of the *D. tsuruhatensis* and *A. xylosoxidans* or, at least, that *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 polymicrobial consortia are more resistant to antibiotic treatment than the corresponding mono-species biofilms (Leriche et al. 2003; Al-Bakri et al. 2005; Burmolle et al. 2006; Kara et al. 2006). This demonstrated that under the challenging conditions imposed by the use of antibacterial agents, the species within a biofilm can cooperate metabolically

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.

in order to protect themselves (Kara et al. 2006; Elias & Banin 2012). Indeed, the population proportions 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 most 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 (Zhanel et al. 2000; Steinman et al. 2003; Hooton et al. 2010; Dellimore et al. 2013). The MBECs were evaluated for single- and dual-species biofilms. The results are listed in 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 would be needed to eradicate the consortia than those required to eradicate single-species biofilms; or, at least, an antibiotic concentration equal to that needed to eradicate the more resistant species which, in this case, were *D. tsuruhatensis* and *A. xylosoxidans*. In general the MBEC results showed the expected behavior. An exception was observed for the *E. coli*/*D. tusuruhatensis* dual-species biofilm where ciprofloxacin was able to eradicate the biofilm at a very low concentration (0.5 mg l^{-1}). While individually the single-species biofilms were highly resistant 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 greatly increased the odds of *E. coli* surviving in the presence of antibiotic agents, it was unclear whether the exposure to these agents resulted in altered population balance. To further investigate this, the dual-species biofilm cells were quantified after exposure to an antibiotic concentration near or below the MBEC (the CFU cm^{-2} values are presented in Supplemental material Tables 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 2A and B). After antibiotic exposure, the results showed that, in general, the relative bacterial composition of the dual-species biofilms was dependent on the antibiotic and the concentration applied (Figure 2). For ciprofloxacin, the three bacteria presented a high MBEC and, thus, the percentages of each population were more balanced. For the other three antibiotics, for which the MBEC values of the uncommon species were much higher than those obtained for *E. coli*, a different behavior was observed. It would be expected that the more resistant species would dominate the microbial consortia. This 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 biofilm cells of the less common species 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 cell

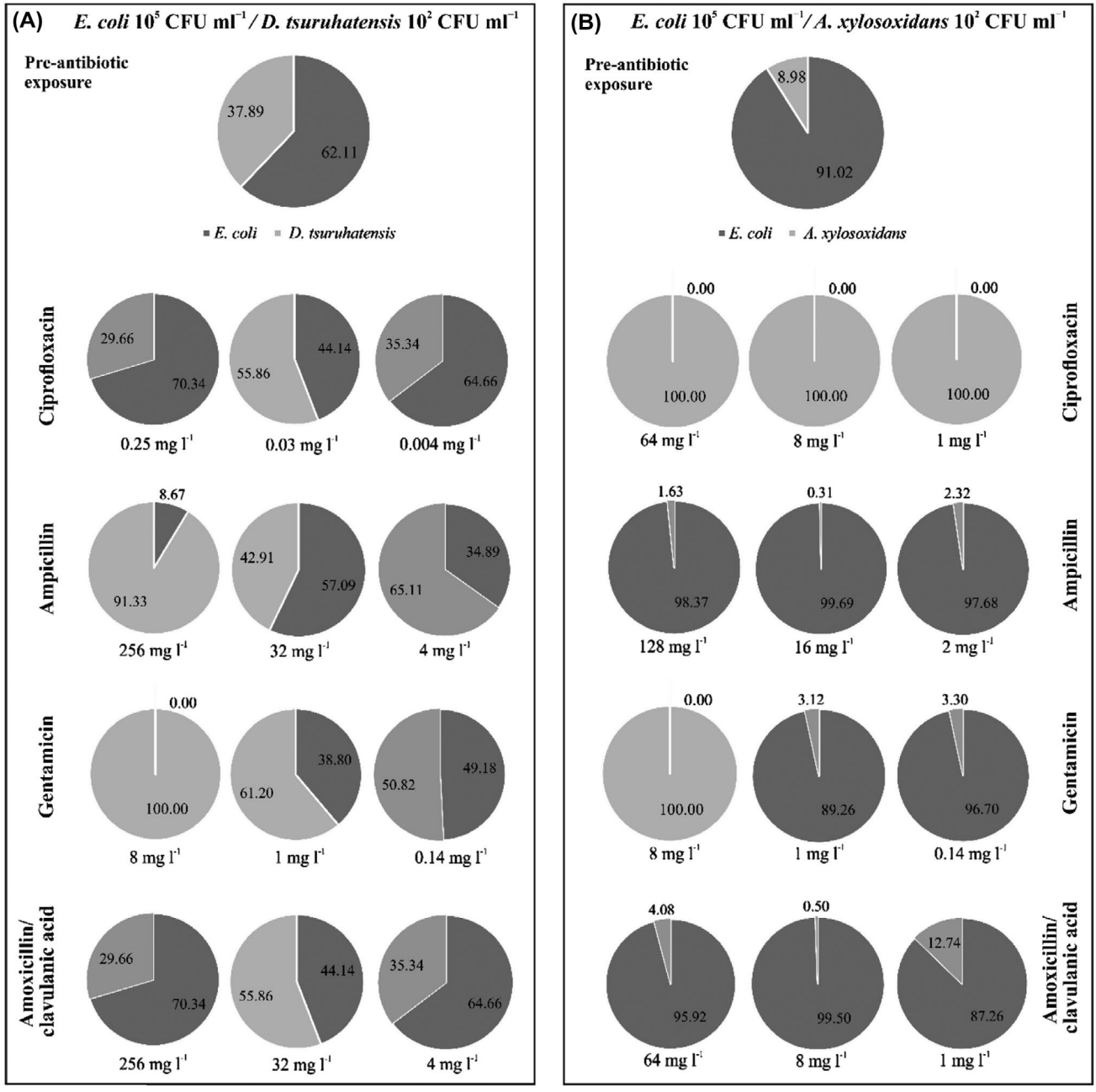

Figure 3. Effect of a lower *D. tsuruhatensis* or *A. xylosoxidans* initial inoculum concentration (10^2 CFU ml^{-1}) on the *E. coli* population after antibiotic exposure.
Notes: For each antibiotic, the *E. coli*/*D. tsuruhatensis* (A) and *E. coli*/*A. xyosoxidans* (B) 48 h dual-species biofilms were 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.

wall biosynthesis in the bacterial cell, which ultimately might lead to cell lysis (Kohanski et al. 2010). Interestingly, biofilm resistance was observed even in the presence of clavulanic acid, which is an inhibitor of 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 to the *E. coli* physiology, promoting its resistance and survival against the ampicillin and amoxicillin/clavulanic acid treatment. To determine whether 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 decreased (to 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.*

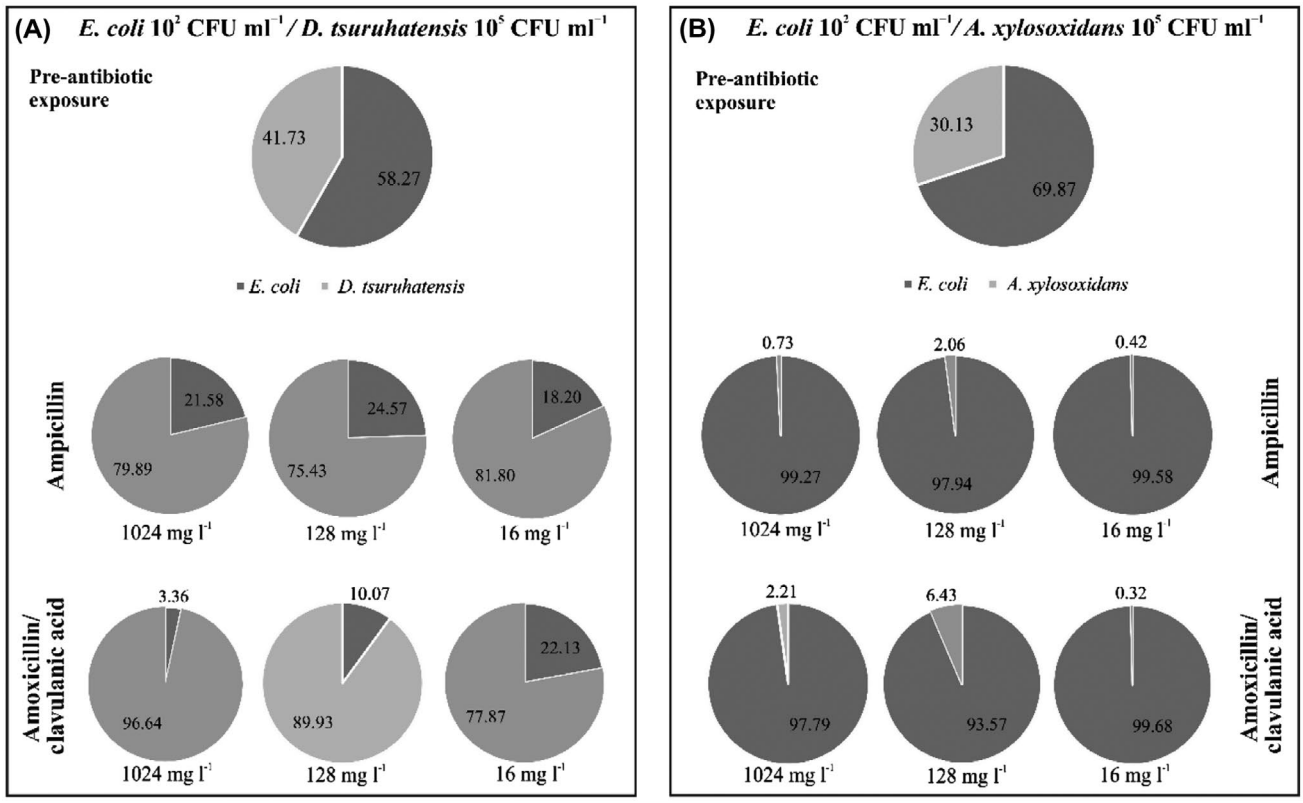

Figure 4. Effect of a lower *E. coli* initial inoculum concentration (10^2 CFU ml^{-1}) on the relative bacterial composition of the dual-species biofilms after exposure to ampicillin and amoxicillin/clavulanic acid.
Notes: For each antibiotic, the *E. coli*/*D. tsuruhatensis* (A) and *E. coli*/*A. xyosoxidans* (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.

coli population dominated the consortia after the antibiotic treatment (Figure 3A and B). The exception was for ciprofloxacin action on *E. coli* 10^5 CFU ml^{-1}/*A. xylosoxidans* 10^2 CFU ml^{-1} (Figure 3B). 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 3A and 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 when its initial inoculum concentration was significantly decreased (10^2 CFU ml^{-1}). The results showed that a lower initial inoculum concentration of *E. coli* did not affect the MBEC value for ampicillin and amoxicillin/clavulanic acid of dual-species biofilms (Table S1). *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 4A). When co-cultured with *A. xylosoxidans*, *E. coli* maintained its dominance (Figure 4B).

The images captured by CLSM revealed that the dual-species biofilms maintained the same structure after treatment with ampicillin (Figure 5). The species are closely associated with a dominance of the *E. coli* population (Figure 5II, VI, VII and VIII). Collectively, these results suggest that the less common species seem to offer a shared resistance to the *E. coli* population, independently of its initial inoculum concentration. Lee et al. (2014) 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.

Recently, several studies have demonstrated the new antibiotic resistance phenomenon of 'shared resistance', involving several members of the microbial consortium (Perlin et al. 2009; Yurtsev et al. 2013; Lee et al. 2014). 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 *E. coli* due to antibiotic uptake; (3) the degradation of the antibiotic in the biofilm matrix, through the action of the β-lactamases produced by the uncommon species.

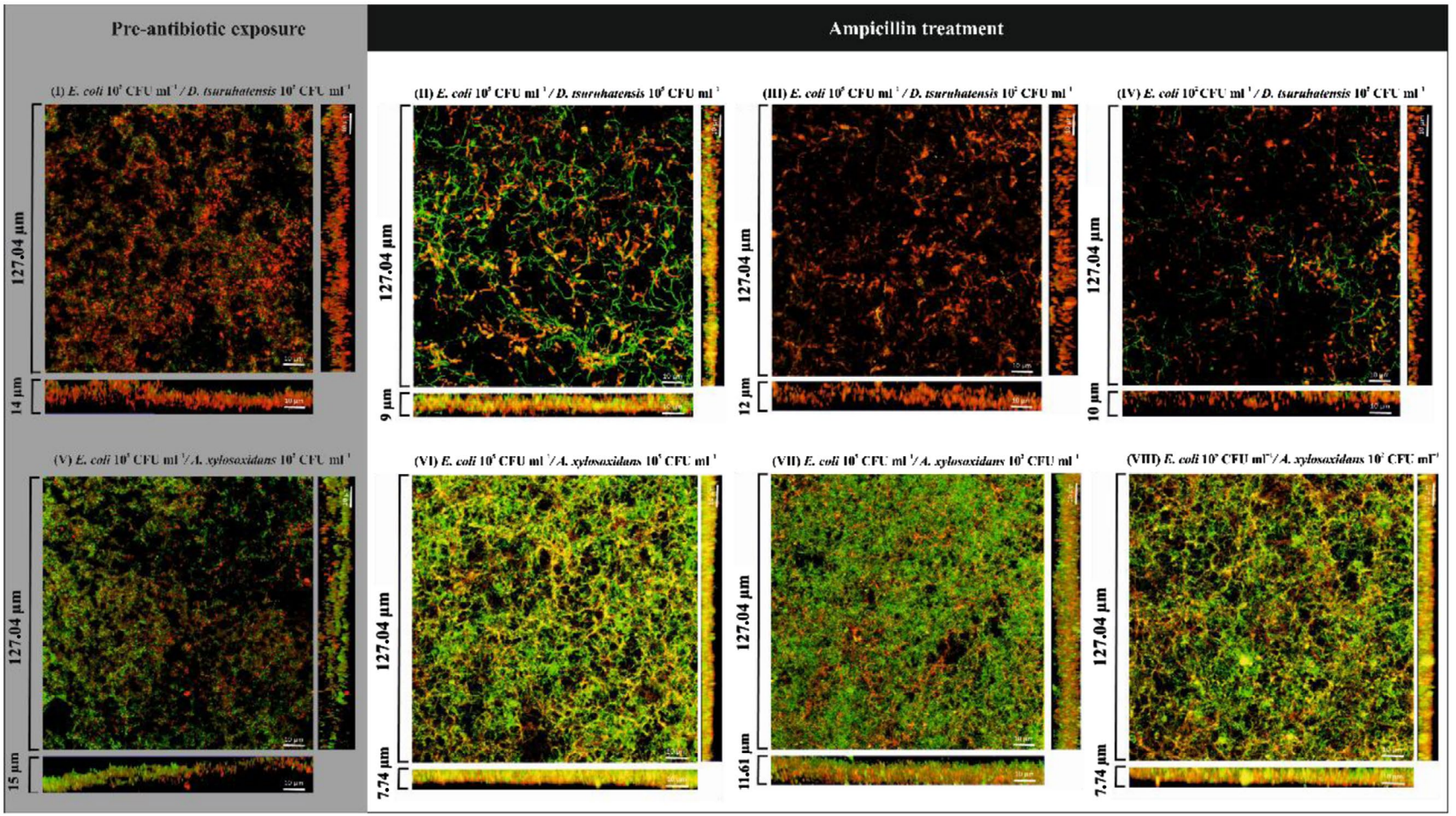

Figure 5. Spatial localization and structure of dual-species biofilms exposed to ampicillin.

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.

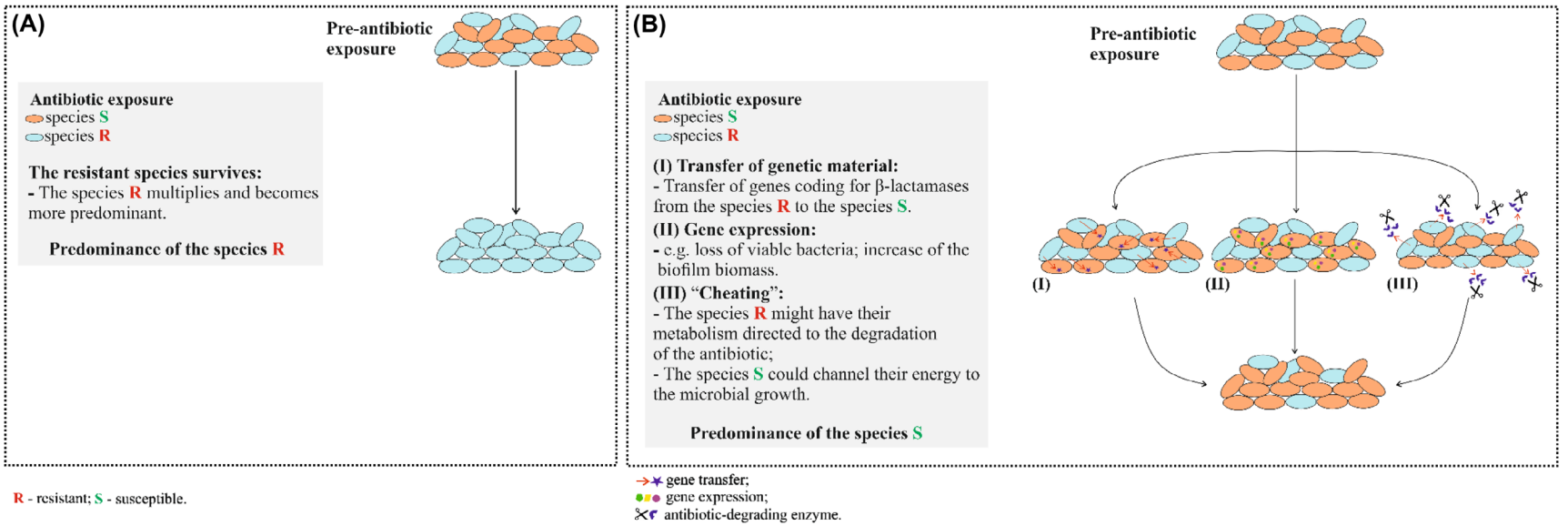

Figure 6. Schematic representation of the hypothesis proposed in the present work to explain the predominance of a certain species when a dual-species biofilm is exposed to antibiotics.

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.

Horizontal gene transfer of antibiotic resistance genes is a process by which some bacteria become resistant to antibiotic agents (Broszat & Grohmann 2014). Recent data have also been provided for the occurrence of this mechanism in biofilms (Ghigo 2001; May et al. 2009; Hoiby et al. 2010; Savage et al. 2013; Broszat & Grohmann 2014) 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 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 the challenging conditions caused by the use of different antibacterial compounds. Another study reported that sub-inhibitory concentrations of β-lactam antibiotics promote alterations in 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 β-lactam antibiotics (Ciofu & Høiby 2008). Lee et al. (2014) suggested that *Pseudomonas protegens* was able to protect all species involved in 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 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 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 (2011), 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 to 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 is preferred. Human urine varies significantly in terms of pH and composition 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 modeling the growth and attachment of urinary pathogens in the clinical environment) (Brooks & Keevil 1997; Klinth et al. 2012; Almeida et al. 2013; Cerqueira et al. 2013; Azevedo et al. 2014). Also, the use of a dynamic biofilm system might allow better mimicking of the urine flow that occurs in the urinary catheter, but the experiments were performed in static conditions using the well plates. These platforms offer the possibility of providing a larger amount of data (Kumar et al. 2004; Duetz 2007).

Conclusions

Interactions between *E. coli* and other less common species in CAUTIs can promote survival of *E. coli* under challenging conditions, such as those imposed by antibiotic agents. A residual concentration of these less common species appears to be sufficient to protect the *E. coli* population. In 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 to explain the predominance of a certain species (*E. coli*, in this case) when a dual-species biofilm is exposed to antibiotic agents or other molecules.

While synergistic interactions between *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 to the role of uncommon species that should be investigated, such as how the protection offered by these species contributes to the survival and dominance of sensitive species under lethal antibiotic concentrations. More experiments involving these types of species should be carried out, and the mechanisms involved in evolution of antibiotic resistance should be taken into consideration. In addition, it is suggested that the microbial composition and environmental conditions present in the polymicrobial biofilms should be considered in the development and validation of novel antimicrobial strategies.

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Disclosure statement

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