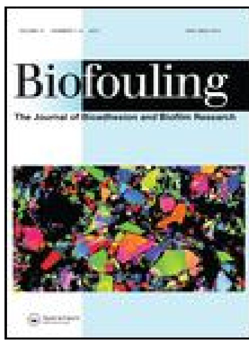

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# Role of interspecies interactions in dual-species biofilms developed *in vitro* by uropathogens isolated from polymicrobial urinary catheter-associated bacteriuria

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**ABSTRACT**
Most catheter-associated urinary tract infections are polymicrobial. Here, uropathogen interactions in dual-species biofilms were studied. The dual-species associations selected based on their prevalence in clinical settings were *Klebsiella pneumoniae–Escherichia coli*, *E. coli–Enterococcus faecalis*, *K. pneumoniae–E. faecalis*, and *K. pneumoniae–Proteus mirabilis*. All species developed single-species biofilms in artificial urine. The ability of *K. pneumoniae* to form biofilms was not affected by *E. coli* or *E. faecalis* co-inoculation, but was impaired by *P. mirabilis*. Conversely, *P. mirabilis* established a biofilm when co-inoculated with *K. pneumoniae*. Additionally, *E. coli* persistence in biofilms was hampered by *K. pneumoniae* but not by *E. faecalis*. Interestingly, *E. coli*, but not *K. pneumoniae*, partially inhibited *E. faecalis* attachment to the surface and retarded biofilm development. The findings reveal bacterial interactions between uropathogens in dual-species biofilms ranged from affecting initial adhesion to outcompeting one bacterial species, depending on the identity of the partners involved.

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Interspecies interactions; dual-species biofilms; urinary catheters; uropathogens

## Introduction

Most microorganisms live in complex communities, such as polymicrobial biofilms, that represent a favorable scenario for interactions across species boundaries to take place. Bacterial biofilms are microbial communities of cells attached to biotic or abiotic surfaces, and embedded in a self-produced extracellular polymeric matrix (Donlan & Costerton 2002). Additionally, biofilms have been characterized by some level of bacterial organization, including signaling in response to the number and identity of cells present and the surroundings (Parsek & Greenberg 2005). Biofilm development involves three distinct events: attachment of cells to a surface, growth of the cells into a sessile biofilm colony, and detachment of the cells from the colony into the surrounding medium. Each of these stages can be shaped by interspecies interactions, ranging from synergistic relationships to bacterial competition (Burmølle et al. 2014). The significance of interspecies interactions has been extensively described for bacteria residing in the oral cavity (Kolenbrander et al. 2010) and also in other natural and artificial environments such as soil, seawater, and drinking water distribution systems, among others (Burmolle et al. 2006; Schwering et al. 2013). These studies indicate the variety and complexity of specific interspecies interactions that could impact multispecies biofilms.

Polymicrobial biofilms have been also found in medical biomaterials and devices, particularly in urinary catheters. It has been reported that up to 86% of the catheter-associated urinary tract infections (CAUTIs) are polymicrobial, usually involving combinations of uropathogenic *Escherichia coli*, *Enterococcus* spp., *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Candida albicans* (Warren et al. 1982; Holá et al. 2010). CAUTI arises through bacterial entry into the urinary tract *via* the urinary catheter. Then, biofilm formation on the catheter surface perpetuates the bacterial presence in the urinary tract (Maki & Tambyah 2001). Risk factors for developing a CAUTI include prolonged catheterization, female gender, older age, and diabetes (Chenoweth et al. 2014). CAUTI and septicemia originating from the urinary tract account for enormous costs for health-care systems, since urinary catheterization is frequent in hospitals and long term care facilities and bacterial colonization cannot be effectively cleared until removal of the catheter (Warren

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2001; Foxman 2014). In addition, the colonized catheter is a source of potential antibiotic resistant bacteria in an environment with susceptible hosts.

Knowledge of interspecies interactions between uropathogens that develop multispecies biofilms on urinary catheters is limited. The urease-positive species *P. mirabilis* has been studied in mixed biofilms in regard to its ability to form crystalline bacterial biofilms that finally results in catheter blockage (Macleod & Stickler 2007). Establishment of mixed biofilms have shown some antagonism between *P. mirabilis* and both *Enterobacter cloacae* and *P. aeruginosa*, but the effects were temporary. Moreover, whatever the pre-existing urinary microbiota was (*E. cloacae*, *P. aeruginosa*, *Morganella morganii*, *E. coli*, or *K. pneumoniae*), colonization with *P. mirabilis* could lead to catheter encrustation and blockage. More recently, a mouse model of ascending UTI was utilized to determine the impact of co-infection between *P. mirabilis* and *Providencia stuartii*, two urease-positive species (Armbruster et al. 2014). Results showed that *P. mirabilis* and *Providencia stuartii* co-infection promotes urolithiasis and bacteremia in a urease-dependent manner, at least in part through synergistic induction of urease activity. On the other hand, it has been reported that co-infection of the urinary tract with *P. mirabilis* and *E. coli* enhanced bacterial colonization and persistence of both pathogens during UTI (Alteri et al. 2015). This result was in agreement with the observed changes in nutritional requirements between *E. coli* and *P. mirabilis* during co-colonization of the urinary tract. Another study addressed the population dynamics of dual-species biofilms composed of *E. coli* and *P. aeruginosa* (Cerqueira et al. 2013). Results indicated that *E. coli* cell numbers decrease when co-cultured with *P. aeruginosa*, but *P. aeruginosa* seems to benefit in dual-species biofilms. Additionally, the coexistance of *E. coli* with two non-pathogenic atypical species (*Delftia tsuruhatensis* and *Achromobacter xylosoxidans*) within dual-species biofilm structures was studied (Azevedo et al. 2014). It was observed that pre-colonization with these atypical species seems to promote adhesion of *E. coli*.

It is well known that different combinations of bacterial species can be found in polymicrobial CAUTIs. The goal of the present study was to expand knowledge on interspecies interactions between uropathogens that develop multispecies biofilms on urinary catheters. Here, four different dual-species associations reported as prevalent in catheter-associated polymicrobial bacteriuria: *K. pneumoniae*–*E. coli* (Kp-Ec), *E. coli*–*E. faecalis* (Ec-Ef), *K. pneumoniae*–*E. faecalis* (Kp-Ef), and *K. pneumoniae*–*P. mirabilis* (Kp-Pm) were examined. The population dynamics of dual-species biofilms developed in artificial urine over a siliconized surface were compared to single-species biofilm development. In addition, for comparative purposes, the effect of co-inoculation in planktonic growth was analyzed. The occurrence of synergistic or competitive interactions in each situation was evaluated.

## Materials and methods

### *Microbiological survey of polymicrobial urine samples from patients with catheter-associated urinary tract infections (CAUTIs)*

A prospective microbiological survey was conducted for the identification of microbial species present in polymicrobial bacteriuria cases from patients with symptomatic CAUTIs. This study was carried out at the Hospital General de Agudos ‘Dr I. Pirovano’ (Buenos Aires, Argentina) between January 2012 and December 2013. Case patients were identified by reviewing all urine culture from patients carrying a urinary catheter reported by the hospital’s microbiology laboratory. A symptomatic polymicrobial CAUTI was defined by the presence of $\geq 10^3$ microorganisms $ml^{-1}$ of two or more bacteria or yeast from the urine of a patient with one or more of the following signs and symptoms and no other identifiable cause: suprapubic tenderness, pelvic discomfort, flank pain, rigors, gross hematuria, delirium, or fever (temperature of at least 100°F). Demographic information of age, sex, duration of catheterization, and microbiology data were collected from the medical records.

The identification and antimicrobial susceptibility of bacterial strains were determined as part of the routine work at the hospital′s microbiology laboratory. Briefly, urine samples were collected aseptically by needle aspiration of the distal catheter and were inoculated onto blood agar, MacConkey’s agar, and cystine lactose electrolite deficient (CLED) agar with calibrated loops to determine CFUs. Each colony of concentration $\geq 10^3$ CFU $ml^{-1}$ that was of distinct morphology was isolated and identified by using conventional biochemical tests. Confirmation of microbial identification and antibiotic susceptibility testing were performed by the automated Vitek-2 system (bioMérieux, Craponne, France).

### *Bacterial strains and inoculum preparation*

Clinical strains were co-isolated from polymicrobial urine samples of individuals undergoing long-term indwelling catheterization and showing symptoms of a urinary infection at Hospital Pirovano, as described above. Isolates corresponding to microbial associations showing higher prevalence were selected (Table 1). The isolates were maintained in the laboratory as frozen stocks (at −80°C) in LB medium supplemented with 15% glycerol. Inocula for both biofilm formation assays and planktonic growth

Table 1. Clinical strains isolated from catheter-associated polymicrobial bacteriuria used in this study.

| Strain | Description | Antibiotic resistance[a] | Source |
|---|---|---|---|
| *Klebsiella pneumoniae* | | | |
| Kp01 | Strain co-isolated with *E. coli* (Ec01) from patient #05 | Ap | This study |
| Kp03 | Strain co-isolated with *E. faecalis* (Ef03) from patient #06 | Ap, CTX, CZD, CIP, TMP/SMX | This study |
| Kp04 | Strain co-isolated with *P. mirabilis* (Pm04) from patient #25 | Ap | This study |
| *Escherichia coli* | | | |
| Ec01 | Strain co-isolated with *K. pneumoniae* (Kp01) from patient #05 | Rf | This study |
| Ec02 | Strain co-isolated with *E. faecalis* (Ef02) from patient #62 | Ap, CIP, CAM TMP/SMX, Tc | This study |
| *Enterococcus faecalis* | | | |
| Ef02 | Strain co-isolated with *E. coli* (Ec02) from patient #62 | CIP, Tc, GEN, Km | This study |
| Ef03 | Strain co-isolated with *K. pneumoniae* (Kp03) from patient #06 | CIP, Tc, GEN, Km | This study |
| *Proteus mirabilis* | | | |
| Pm04 | Strain co-isolated with *K. pneumoniae* (Kp04) from patient #25 | CIP, TMP/SMX, Tc, Km, CAM | This study |

[a]Ap, ampicillin; CTX, cefotaxime; CZD, ceftazidime; CIP, ciprofloxacin; TMP/SMX, trimethoprim/sulfamethoxazole; Rf, rifampicin; Tc, tetracycline; CAM, chloramphenicol; GEN, gentamycin; Km, kanamycin.

were prepared as follows. For each experiment, clinical strains were freshly streaked in LB-agar plates and grown overnight at 37°C. Subsequently, individual colonies from each strain were used to inoculate LB medium and cells were incubated overnight at 37°C and 200 rpm. Then, each inoculum was diluted in artificial urine medium (AUM) (Brooks & Keevil 1997) in order to obtain a final concentration of $3 \times 10^7$ cells ml$^{-1}$ (single-species inoculum). For dual-species inoculum, equal volumes of each single-species inoculum in AUM were mixed. The bacterial concentration used as starting inoculum was similar to what was used by other authors when performing competition experiments between uropathogens in AUM (Cerqueira et al. 2013; Armbruster et al. 2014; Alteri et al. 2015; Lehman & Donlan 2015). Note that no antibiotics were added to any of the bacterial inocula.

### Biofilm formation assays

A total of 3 ml of each single culture inoculum ($3 \times 10^7$ CFU ml$^{-1}$ in AUM) was transferred to a 50-ml Falcon tube and a sterile 22 mm-diameter siliconized coverslip (Hampton Research, Aliso Viejo, CA, USA) was placed over the bacterial suspension. For dual-species experiments, equal volumes of each single culture were first mixed and, subsequently, 3 ml of the final inoculum were used for performing the assays. The tubes were incubated statically at 37°C. Adhesion to siliconized coverslips was allowed to occur for 3 h. At 3 h post-inoculation, and every 24 h, the AUM was removed, coverslips were washed three times with sterile 0.9% NaCl solution, and the medium was replaced by fresh AUM. At selected time points, an aliquot of the AUM surrounding the biofilm was saved, then biofilms were washed three times with sterile 0.9% NaCl solution, and adhered biofilm was mechanically disrupted from the coverslip by scraping. The number of cultivable cells in the biofilms and in the AUM surrounding the biofilm was determined (see 'Quantification of cultivable cells').

### Planktonic growth

A total of 3 ml of each single- and dual-species inoculum ($3 \times 10^7$ cells ml$^{-1}$ in AUM), prepared as described in 'inoculum preparation', was monitored for static planktonic growth at 37°C. When indicated, AUM was buffered to pH 6.5 by addition of 100 mM MES [2-(*N*-morpholino) ethanesulfonic acid] (Sigma-Aldrich, St Louis, MO, USA). Cultures were sampled at the indicated time points to determine CFU ml$^{-1}$ as described in 'Quantification of cultivable cells'. To monitor the pH of *P. mirabilis* cultures, an aliquot of the culture was centrifuged at $5,000 \times g$, and the pH of the cell-free supernatant was measured using a digital pH-meter (Orion 3 star Thermo Scientific, Beverly, MA, USA).

### Quantification of cultivable cells

The number of cultivable cells from disrupted biofilms, from planktonic bacteria in the surrounding biofilm or from planktonic culture was determined by counts of colony forming units (CFUs.). For this purpose, bacteria were serially diluted (1:10) and plated on LB-agar plates containing appropriate antibiotics. For *P. mirabilis* CFUs counts were done on LB-agar plates which contained 10-fold less NaCl to avoid *P. mirabilis* swarming (Pearson et al. 2010). In order to distinguish between the two bacterial species in the dual-species experiments, the antibiotic susceptibility of each strain to a number of antibiotics was determined by agar dilution method (Masuda & Tomioka 1978). Ec01 was found to be resistant to $\geq 100$ μg ml$^{-1}$ rifampicin (Rf) and susceptible to $> 10$ μg ml$^{-1}$ ampicillin (Ap) while Kp01 was sensitive to 10 μg ml$^{-1}$ Rf and resistant to $\geq 100$ μg ml$^{-1}$ Ap; therefore,

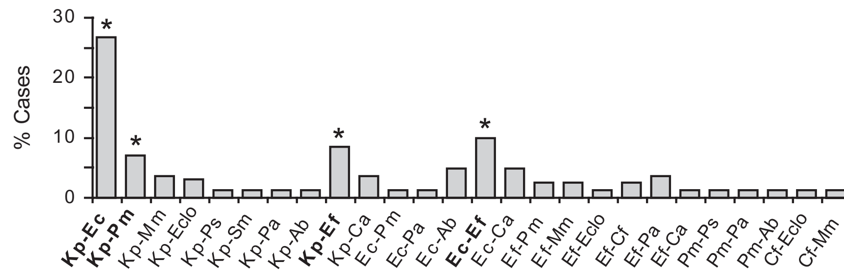


**Figure 1.** Frequency of microorganisms co-isolated from urine of patients carrying a urinary catheter.
Notes: Results from a 2012–2013 laboratory-based surveillance study in a public hospital from Buenos Aires city, Argentina. Bacterial species found in patients with catheter-associated polymicrobial bacteriuria were *Klebsiella pneumoniae* (Kp), *Escherichia coli* (Ec), *Proteus mirabilis* (Pm), *Morganella morganii* (Mm), *Enterobacter cloacae* (Eclo), *Providencia stuartii* (Ps), *Serratia marcescens* (Sm), *Pseudomonas aeruginosa* (Pa), *Acinetobacter baumanii* (Ab), *Enterococcus faecalis* (Ef), *Candida albicans* (Ca), and *Citrobacter freundii*. Asterisks (*) indicate the four prevalent associations: Kp-Ec; Kp-Pm; Kp-Ef; Ec-Ef.

LB-agar plates were supplemented with Rf (50 μg ml$^{-1}$) or Ap (30 μg ml$^{-1}$) to distinguish between these two species. Ec02 was determined to be resistant to > 100 μg ml$^{-1}$ Ap and sensitive to ≥ 10 μg ml$^{-1}$ kanamycin (Km) whereas Ef02 was sensitive to ≥ 10 μg ml$^{-1}$ Ap and resistant to ≥ 100 μg ml$^{-1}$ Km; consequently, LB- agar plates were supplemented with Ap (30 μg ml$^{-1}$) or Km (30 μg ml$^{-1}$) to distinguish between Ec02 and Ef02. Kp03 was found to be resistant to ≥ 100 μg ml$^{-1}$ Ap and sensitive to ≥ 1 μg ml$^{-1}$ Km while Ef03 presented similar antibiotic resistance profile to Ap and Km than Ef02; thus, LB- agar plates were supplemented with Ap (30 μg ml$^{-1}$) or Km (30 μg ml$^{-1}$) to distinguish between Kp03 and Ef03. Kp04 showed resistance to ≥ 100 μg ml$^{-1}$ Ap and sensitivity to 1 μg ml$^{-1}$ tetracycline (Tc) while Pm04 was sensitive to ≥ 1 μg ml$^{-1}$ Ap and resistant to ≥ 50 μg ml$^{-1}$ Tc; therefore, LB- agar plates were supplemented with Ap (30 μg ml$^{-1}$) or Tc (10 μg ml$^{-1}$) to distinguish between these two species.

### *Statistical analysis*

Statistical significance was assessed using one-way analysis of variance (ANOVA) with Bonferroni post-test. A $p$-value <0.05 was considered significant. Analyses were performed using GraphPad Prism, version 4 (GraphPad Software, San Diego, CA, USA).

## Results and discussion

### *Microbial species co-isolated in polymicrobial urine samples*

Biofilms formed in medical devices, such as urinary catheters, are usually polymicrobial (Holá et al. 2010). Few studies have focused on the process of multispecies biofilm formation in urinary catheters and the contribution of each population to this process (Macleod & Stickler 2007; Azevedo et al. 2014). Here, to identify the microbial species most commonly present together in polymicrobial catheter-associated urinary tract infections (CAUTIs), a two-year prospective microbiologic survey of polymicrobial bacteriuria in patients with CAUTI was performed at a public hospital from Buenos Aires, Argentina. Of the 97 polymicrobial cases, most of the patients were elderly men/woman (73 ± 10 years old) with a urinary catheter in place for a mean time of 41 days (Supplemental material, Table S1). Two and three microbial species were co-isolated in 96% and 4% of the samples, respectively. The following microorganisms were detected in at least one sample: *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Providencia stuartii*, *Acinetobacter baumanii*, *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens*, *Enterococcus faecalis,* and *Candida albicans* (Figure 1). The dual-species associations showing higher prevalence in these polymicrobial urine samples were *K. pneumoniae*–*E. coli* (Kp-Ec), *E. coli*–*E. faecalis* (Ec-Ef), *K. pneumoniae*–*E. faecalis* (Kp-Ef), and *K. pneumoniae*–*P. mirabilis* (Kp-Pm) accounting for 26, 10, 8.5, and 7% of the cases, respectively. Whereas several reports have shown that bacteria isolated from urine are not necessarily the same as those found in urinary tract-associated biofilm (Farsi et al. 1995; Djeribi et al. 2012), other studies have shown cases of identical microorganisms in catheter and urinary cultures (Yeniyol et al. 2002; Matsukawa et al. 2005). Particularly, regarding long-term catheterized individuals, it has been reported that the percentage of patients who had the same bacterial species isolated from both specimens (catheter and urine) increased in a time-dependent manner and reached > 80% at day 14 or later (Matsukawa et al. 2005). This is relevant to the present study because the polymicrobial bacteriuria cases analyzed here corresponded to patients carrying a urinary catheter for at least 20 days (20–126 days; Table S1). Thus, it is very likely the bacterial populations isolated from the urine cultures in this study were also co-colonizing the catheter surfaces. Similarly, other authors have reported that catheter infections of urinary tracts are caused most

**Table 2.** Bacterial attachment to siliconized surface.

| Strain | No. of attached cells ($10^4$ CFU $cm^{-2}$) | |
|---|---|---|
| | Pure inoculation | Co-inoculation[a] |
| Kp01 | 12.1 ± 0.9 | 8.3 ± 1.5 |
| Kp03 | 5.9 ± 0.6 | 5.3 ± 1.5 |
| Kp04 | 5.5 ± 0.2 | nd[b] |
| Ec01 | 9.3 ± 0.8 | 8.6 ± 4.4 |
| Ec02 | 4.4 ± 0.9 | 4.0 ± 1.7 |
| Ef02 | 2.0 ± 0.4 | 0.19 ± 0.04 |
| Ef03 | 2.0 ± 0.3 | 1.2 ± 0.2 |
| Pm04 | 4.2 ± 0.9 | 6.0 ± 1.6 |

[a]Co-inoculations were as follows: Kp01-Ec01; Ec02-Ef02, Kp03-Ef03; Kp04-Pm04.
[b]nd = not detected (detection limit in this assay was 30 CFU $cm^{-2}$).

commonly by combinations of Gram-negative rods (eg *E. coli*, *K. pneumoniae*, *Enterobacter* sp., *P. aeruginosa*, and *P. mirabilis*) and enterococci (especially *E. faecalis*); however, no discrimination between the specific pathogens found together was made (Warren 2001; Holá et al. 2010; Djeribi et al. 2012). Moreover, the prevalent dual-species associations found here in polymicrobial urine samples of individuals with CAUTIs (Kp-Ec; Ec-Ef; Kp-Ef; Kp-Pm) have been reported together in urinary catheters (Ganderton et al. 1992; Desai et al. 2001; Macleod & Stickler 2007; Frank et al. 2009; Wang et al. 2010). Notably, these bacteria are often highly antimicrobial resistant (2014). Indeed, the microbiological survey performed here revealed that 32% and 55% of the *E. coli* and *K. pneumoniae* isolates, respectively, were extended spectrum beta-lactamase producing microorganisms. Additionally, 12% of *K. pneumoniae* isolates were carbapenemase producing bacteria. These findings raise concerns about the possibility that colonized catheters might be a source of antibiotic resistant bacteria in an environment with susceptible hosts.

To investigate the role of interspecies interactions in dual-species biofilms developed by microorganisms present in polymicrobial CAUTIs, one representative pair of clinical strains, corresponding to each of the four prevalent dual-species associations found (Kp01-Ec01; Kp04-Pm04; Kp03-Ef03; Ec02-Ef02), were selected for further *in vitro* studies.

### *Biofilm formation and planktonic growth of single-species cultures in artificial urine*

In order to assess biofilm formation capabilities of the individual isolates under study, selected *K. pneumoniae*, *E. coli*, *E. faecalis*, and *P. mirabilis* clinical strains were inoculated in artificial urine medium (AUM) on a siliconized surface to partially mimic the medium and surface encountered by bacteria in urinary catheters. Initial attachment and biofilm development was assessed by determining the number of cultivable cells, after mechanically disrupting the attached bacteria. Data from Table 2 (see pure inoculation column) showed that all clinical isolates under study were able to attach to the siliconized surface, although variable numbers of bound cells (2–12 × $10^4$ cells $cm^{-2}$) were detected depending of the particular strain analyzed. Moreover, all these clinical isolates developed single-species biofilms that maintained relatively stable populations after five days in monoculture (Figures 2A and S1). Both single-species biofilms of *K. pneumoniae* and *E. coli* biofilms showed significantly higher cell numbers than either *E. faecalis* or *P. mirabilis* biofilms. Similarly, other studies have also demonstrated the ability of *K. pneumoniae*, *E. coli*, *P. mirabilis*, and *E. faecalis* to form single-species biofilms on silicone, using urine as a growth medium (Ferrieres et al. 2007; Stahlhut et al. 2012).

It was also pertinent to analyze, for comparative purposes, how these clinical strains grew planktonically in AUM when inoculated individually. As expected, in liquid cultures both *K. pneumoniae* and *E. coli* strains grew well, reaching ~$10^8$ CFU $ml^{-1}$ after 4 h static culture at 37°C (Figures 2B and S2). This result is in agreement with the reported bacterial growth of up to ~$10^8$ CFU $ml^{-1}$ in the AUM formulation used in this study which is similar to the levels found in normal urine (Brooks & Keevil 1997). Both *E. faecalis* and *P. mirabilis* cultures grew to a maximal cell density of ~6 × $10^7$ CFU $ml^{-1}$ after 4 h culture. Moreover, *P. mirabilis* was the only bacterial species whose viable cell number declined over time in AUM (fivefold decrease after 24 h). It is well known that *P. mirabilis* expresses a urease that hydrolyses urea and catalyzes formation of ammonium and carbon dioxide (Armbruster & Mobley 2012). The AUM formulation has a high urea content (170 mM); consequently, *P. mirabilis* growth in AUM caused a gradual increase in medium pH, from 6.5 (pH of AUM) to ~9.0 after 6 h culture (Figure S3A). It seems that this high pH self-limited *P. mirabilis* growth because when the pH was maintained around 6.5, either by removing the urea from the AUM formulation or by buffering the medium with MES, the decrease in *P. mirabilis* viable cell number was not observed and instead a 10-fold cell number increase with respect to the initial inoculum occurred (Figure S3B). A similar decline in *P. mirabilis* growth in an artificial urine medium has been reported (Armbruster et al. 2014) and this effect was attributed to the urease-dependent alkalinization of the urine since no decline in cell numbers was observed for a *P. mirabilis* mutant strain producing an inactive urease.

After studying monospecies cultures, the next step was to determine whether interactions in mixed inoculations had an impact on bacterial biofilm formation and planktonic growth. The focus was on investigating the four dual-species combinations found to be prevalent in catheter-associated polymicrobial bacteriuria: Kp-Ec; Kp-Pm; Kp-Ef; and Ec-Ef.

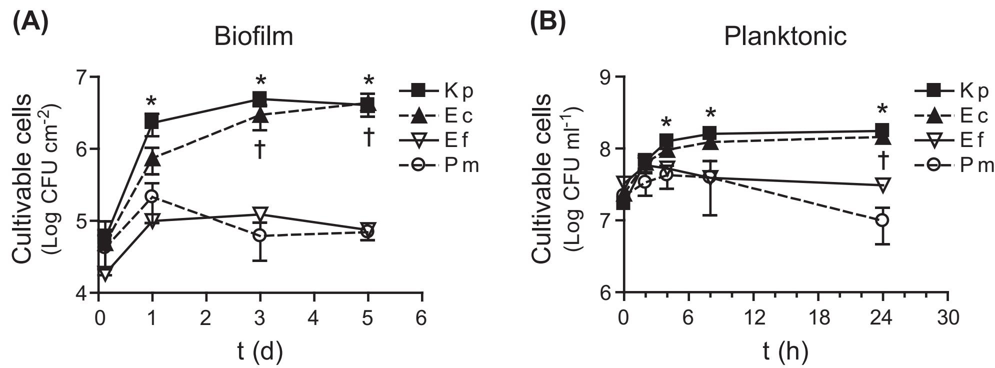


**Figure 2.** Behavior of single-species bacterial growth in biofilms and planktonically.
Notes: (A) Time-course of biofilm formation in AUM assessed as the number of cultivable cells $cm^{-2}$ recovered from siliconized coverslips. (B) Time-course of planktonic growth in AUM by determining the number of cultivable cells. Three independent experiments were performed for each strain and the results were averaged by bacterial species [*K. pneumoniae* (Kp): Kp01, Kp03, Kp04; *E. coli* (Ec): Ec01, Ec02; *E. faecalis* (Ef): Ef02, Ef03; *P. mirabilis* (Pm): Pm04]. Error bars represent the SD. The results for each individual strain are shown in Figures S1 and S2 for biofilm formation and planktonic growth, respectively. (*) $p<0.05$ for Kp *vs* Ef and Kp *vs* Pm; (†) $p<0.05$ for Ec *vs* Ef and Ec *vs* Pm.

### *Detrimental effect on* E. coli *in* K. pneumoniae–E. coli *dual-species biofilms*

To study whether interspecies interactions between *K. pneumoniae* and *E. coli* modulate their biofilm formation ability, *K. pneumoniae* Kp01 and *E. coli* Ec01 clinical strains were co-inoculated in AUM in a 1:1 mixture and biofilm formation was monitored. In mixed inoculations, both species attached to the siliconized surface to similar levels of that in pure cultures (Table 2). Comparisons between single and dual biofilm development showed a significant reduction in the number of cultivable *E. coli* cells in dual-species biofilms at day 2 post-inoculation (Figure 3A). The decline in viable *E. coli* cells was maximal at day 3 post-inoculation and was still observed at day 5 (~70-fold lower *E. coli* cells in dual-species biofilms compared to *E. coli* single-species ones). Conversely, the number of *K. pneumoniae* cells was similar in both Kp-Ec dual-species biofilms and single-species *K. pneumoniae* biofilms, at all the time-zpoints analyzed. Similar results were observed when two different pairs of *K. pneumoniae* and *E. coli* clinical isolates were tested (not shown). Considering cells are detached from the biofilms and dispersed into the medium as part of the biofilm life cycle (Boles et al. 2005), it could be possible that Kp-Ec dual-species biofilm favored *E. coli* detachment. To test this hypothesis, quantification of cultivable planktonic cells in the AUM covering the biofilms was performed. In single-species biofilms, both *K. pneumoniae* and *E. coli* strains had ~ 0.5–2 × $10^8$ cell $ml^{-1}$ in suspension at all the time-points tested. In Kp-Ec dual-species biofilms, the amount of viable dispersed *E. coli* cells was lower than in single-species biofilm, showing a marked decrease (~100-fold) since day 2 and up to day 5 post-inoculation (Figure 3B). Therefore, it seems that Kp-Ec dual-species biofilms did not favor the dispersion of viable *E. coli* cells from the biofilm into the surrounding medium. On the other hand, the number of dispersed *K. pneumoniae* cells from dual-species biofilms was similar to single-species biofilms. Altogether, these results indicate the existence of a detrimental effect of *E. coli* in *K. pneumoniae–E. coli* dual-species biofilms.

To investigate whether this effect also occurs when both bacteria grow together planktonically, evaluation of both *K. pneumoniae* and *E. coli* populations in mixed liquid cultures was performed. Compared to pure cultures, a not statistically significant twofold decrease in *E. coli* growth was detected at 24 h co-culture whereas, as expected, no differences in *K. pneumoniae* growth were observed (Figure 4A). Taken together, these results suggest that the detrimental effect of *K. pneumoniae* over *E. coli* when in co-culture in AUM occurs mainly when both bacterial species are forming a mixed biofilm.

The observation of a differential effect on mixed biofilms, not seen when the respective bacteria are grown planktonically in mixed culture, is in agreement with the reported observation of changes in gene expression associated with the biofilm lifestyle compared to planktonic bacteria (Lazazzera 2005). In this regard, quorum sensing, a sensory system that enables bacteria to detect population densities and regulate gene expression accordingly, has been postulated as important for interspecies interactions including bacterial communication and coordination (Riedel et al. 2001; Parsek & Greenberg 2005; Kolenbrander et al. 2010). It would be possible that novel biofilm gene expression patterns can lead to the production of biofilm-specific metabolites and polymers, some of which could display antagonistic activities against other microorganisms in mixed species context (Ghigo 2003). Additionally, because the biofilm matrix acts as a molecular reservoir due to limited outward diffusion, antagonist molecules released by bacteria into biofilms would be more concentrated in local areas, leading to increased

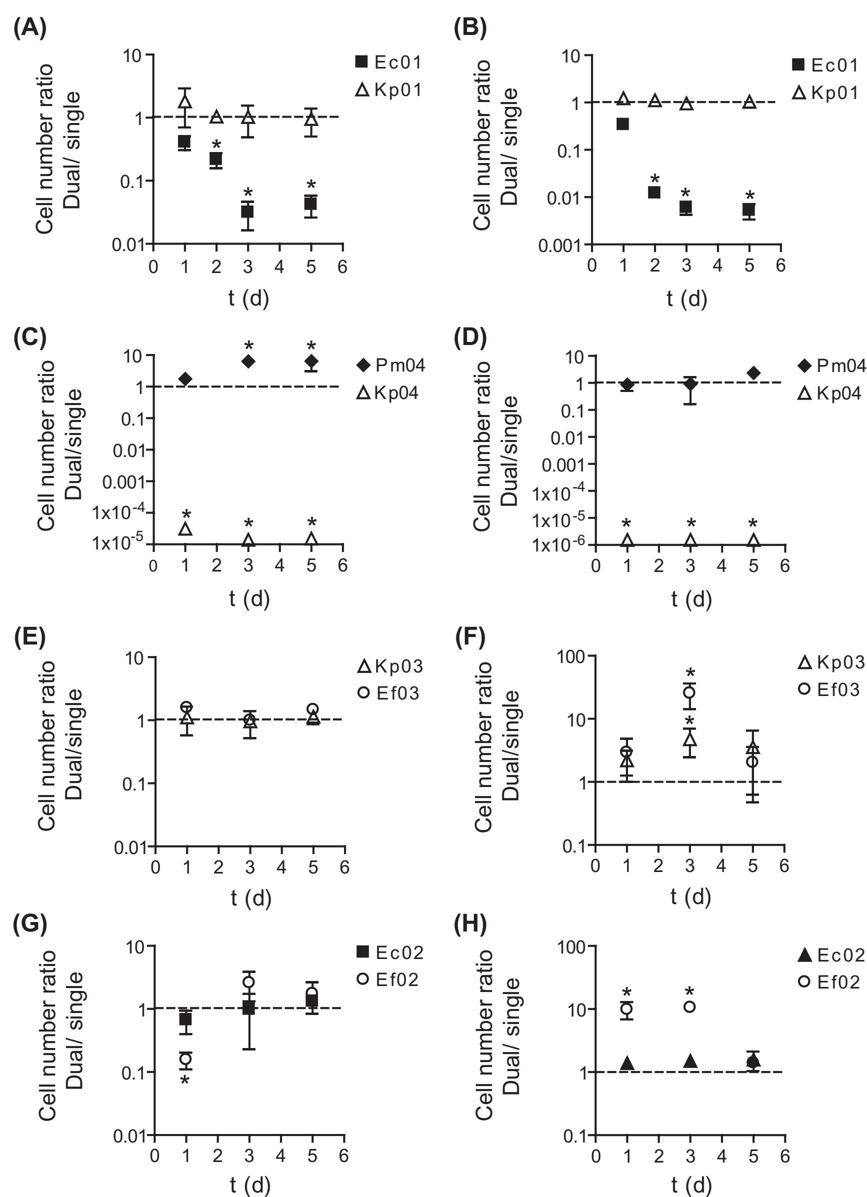


**Figure 3.** Bacterial behavior in dual-species biofilms compared to single-species biofilms.
Notes: Dual-species biofilms analyzed were: Kp01-Ec01 (A, B); Kp04-Pm04 (C, D); Kp03-Ef03 (E, F); Ec02-Ef02 (G, H). Dual to single cell number ratios were calculated for each bacterial species between days 1 and 5. Number of cultivable cells into biofilms (panels A, C, E, G) or dispersed from biofilms (panels B, D, F, H) were assessed as described in legend of Figure 2 or by sampling the AUM suspension in contact with the biofilms, respectively. In all cases, bacteria were plated on selective antibiotics to distinguish between species. Error bars represent means ± SDs of three independent experiments. Dotted lines indicate a dual to single cell number ratio equal to 1, which would represent no differential behavior of a particular strain between dual- and single-species biofilm. (*) $p<0.01$ when compared to the initial ratio (at $t_0$ ratio=1).

efficiency against neighboring competitors (Julou et al. 2013). Further investigations are ongoing to better understand, at the molecular level, the mechanisms responsible for this negative effect on *E. coli*.

## *Co-inoculation of* K. pneumoniae *with* P. mirabilis *affected both* K. pneumoniae *establishment in dual-species biofilms and planktonic growth*

To assess whether inter-species interactions between *K. pneumoniae* and *P. mirabilis* affected their biofilm formation abilities, *in vitro* establishment of Kp04-Pm04 dual-species biofilms was studied. After mixed inoculation, monitoring of initial bacterial attachment to the siliconized surface indicated that *P. mirabilis* was able to adhere to the surface in a similar manner whether in co-culture with *K. pneumoniae* or in pure cultures (Table 2). Conversely, *K. pneumoniae* could not be detected on the surface after 3 h of being co-inoculated with *P. mirabilis*. As described above for monomicrobial *P. mirabilis* planktonic growth in AUM, mixed Kp-Pm co-cultures also showed a gradual increase in pH over time, which

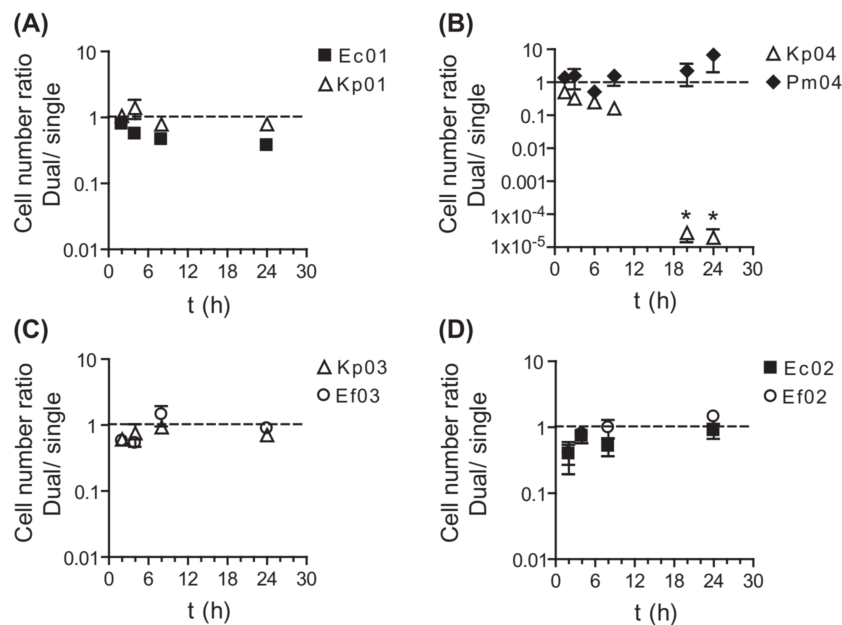


**Figure 4.** Comparison of planktonic growth after single- and dual-species inoculation.
Notes: The bacterial co-cultures analyzed were: Kp01-Ec01 (A); Kp04-Pm04 (B); Kp03-Ef03 (C); Ec02-Ef02 (D). Dual to single cell number ratios were obtained as described in legend of Figure 3. Error bars represent means ± SDs of three independent experiments. Dotted lines indicate a dual to single cell number ratio equal to 1, which would represent no differential behavior of a particular strain between dual- and single-species biofilm. (*) $p<0.01$ when compared to the initial ratio (at $t_0$ ratio=1).

reached a pH value of 7.9 at 3 h post-inoculation. Under this condition, planktonic *K. pneumoniae* growth was limited and, consequently, a 60% reduction in viable *K. pneumoniae* cells was observed when compared to pure cultures ($8.2 \times 10^7$ *vs* $3.4 \times 10^7$ cells $ml^{-1}$) (Figure 4B). This reduction in viable *K. pneumoniae* cells when co-cultured with *P. mirabilis* does not explain the marked inability of *K. pneumoniae* to bind to the siliconized surface. Thus, it is very likely other events took place during the 3 h of Kp-Pm co-culture, where attachment is allowed to occur, that negatively affected the ability of *K. pneumoniae* to bind to the surface. The relevance of the pH change for this *K. pneumoniae* behavior remains to be elucidated. Regarding biofilm development, not surprisingly, viable *K. pneumoniae* cells were almost undetectable after Kp-Pm co-inoculation for one day in both attached biofilm and dispersed cells (Figure 3C and D). Moreover, mixed planktonic cultures showed a time-dependent decrease in *K. pneumoniae* viability when compared to pure liquid cultures of *K. pneumoniae* (Figure 4B). Thus, an ~$10^5$-fold decrease in viable *K. pneumoniae* cells was observed after planktonic co-culture for 20 h. Experiments undertaken with other *K. pneumoniae* and *P mirabilis* clinical isolates provided similar results (not shown). Determination of the medium pH at different time points after Kp-Pm co-inoculation showed that a maximum pH of 9.3 was reached at 12 h post-inoculation (data not shown). Ongoing experiments suggest that this increase in pH is not fully responsible for *K. pneumoniae* cell death. On the other hand, *P. mirabilis* viable cell numbers were neither negatively affected in planktonic mixed cultures nor in dual-species biofilms (Figures 3C, D and 4B). *P. mirabilis* grew to an approximately sixfold greater extent in biofilms formed after inoculation of mixed bacteria than after inoculation of pure *P. mirabilis* cultures, at days 3 and 5 post-inoculation. These results indicate that co-inoculation of *K. pneumoniae* with *P. mirabilis* resulted in a beneficial effect for *P. mirabilis* biofilm development, while impairing *K. pneumoniae* biofilm establishment and planktonic growth. Further investigations will be necessary to better understand the mechanisms behind *K. pneumoniae* and *P. mirabilis* interactions.

### *Interactions between* K. pneumoniae *and* E. faecalis *in dual-species biofilms could favor the presence of detached planktonic cells at the biofilm surroundings*

Biofilm interspecies relationships between *K. pneumoniae* and *E. faecalis* were studied using the co-isolated clinical strains Kp03 and Ef03. Neutral interactions were apparent for Kp-Ef biofilm formation, since both initial attachment and biofilm development (measured as viable cell numbers) of each individual species were similar when mixed *vs* pure inoculation were compared (Table 2 and Figure 3E). Surprisingly, an increased number of dispersed viable cells (10-fold and a 25-fold higher amount of detached

*K. pneumoniae* and *E. faecalis* cells, respectively) were detected in the surrounding medium of dual-species biofilms with respect to single-species biofilms at day 3 post-inoculation (Figure 3F). To evaluate the hypothesis that mixed bacteria growing planktonically interact in a synergistic manner, and consequently improve growth of both *K. pneumoniae* and *E. faecalis* in the surrounding medium after detachment from the biofilm, liquid Kp-Ef co-cultures in AUM were analyzed over 24 h. Figure 4C showed that no significant differences in planktonic growth were observed for planktonic Kp-Ef co-cultures in AUM when compared to the corresponding pure cultures. Taken together, these results suggest that establishment of dual-species biofilms by *K. pneumoniae* and *E. faecalis* could increase the number of daughter cells originating from bacterial biofilm shedding.

The cooperative growth behavior observed for dispersed cells from mixed Kp-Ef biofilms could be the result of changes in gene expression in these bacterial strains that occurred specifically as a consequence of inter-bacterial species communication in mixed biofilm (Lazazzera 2005). Further study of the factors involved in this cooperative effect between *K. pneumoniae* and *E. faecalis* should improve our understanding of the interactions between these uropathogens.

### *Decrease of* E. faecalis *attachment on siliconized surfaces when co-inoculated with* E. coli

The existence of bacterial interactions in dual-species biofilms formed by *E. coli* and *E. faecalis* was investigated. For this purpose, the clinical co-isolated strains *E. coli* Ec02 and *E. faecalis* Ef02 were utilized. Ef02 strain, but not the Ec02 strain, showed a lower initial attachment (a 10-fold difference) when co-inoculated compared to that in pure inoculation (Table 2). Moreover, a 10-fold decrease in *E. faecalis* cell number was found in one day-old Ec-Ef dual-species biofilms, in comparison to *E. faecalis* single-species biofilms (Figure 3G). However, this difference was not observed at day 3 or day 5. Conversely, no effect of either *E. coli* initial attachment or biofilm formation was observed when *E. coli* was co-inoculated with *E. faecalis*. These results suggest that *E. faecalis* attachment was outcompeted by *E. coli*, causing an initial delay in *E. faecalis* growth in dual-species biofilms. Nonetheless, this effect could be overcome by prolonged incubation. It has been reported that certain *E. coli* isolates inhibited biofilm formation by Gram-positive bacteria, including *E. faecalis*, by secreting polysaccharides that alter the properties of abiotic surfaces (Rendueles et al. 2011). Whether this occurs with the clinical *E. coli* strain used here remains to be determined. A 10-fold increase in the number of *E. faecalis* cells, but not *E. coli* cells, was found in the medium surrounding the biofilm on day 1 and day 3 post-inoculation (Figure 3H). Further assessment of whether *E. faecalis* benefited when grown with *E. coli* in planktonic cultures was performed. Figure 4D shows that neither *E. faecalis* nor *E. coli* showed any differential growth behavior in either pure or mixed cultures. This result suggests that Ef-Ec dual-species biofilms somehow favored dispersal of viable cells of *E. faecalis* into the medium, similar to what was observed in Kp-Ef biofilms.

## Conclusions

This work examined the interspecies interactions of a set of chosen uropathogens shown to be prevalent in polymicrobial CAUTIs (Kp-Ec, Kp-Pm, Kp-Ef, and Ec-Ef). Bacterial viability was assessed over time to determine both planktonic and biofilm microbial population composition. Thus, for the planktonic mode of growth, no differences were observed in individual species population density which originated from dual-species cultures as compared to single-species cultures. The only exception was Kp-Pm, where a significant decline in *K. pneumoniae* viability occurred as a consequence of co-inoculation. With respect to the biofilm mode of growth, a variety of scenarios affecting different steps of biofilm formation was observed as a consequence of co-inoculations, depending on the bacterial species. For instance, *E. faecalis* was negatively affected in terms of attachment to the surface when it was co-inoculated with *E. coli*, and, furthermore, a substantial impairment of initial attachment of *K. pneumoniae* biofilm occurred when this bacterial species was co-inoculated with *P. mirabilis*. On the other hand, in Kp-Ec dual-species biofilms, an effect on biofilm development was observed, with a marked decline in the number of viable *E. coli* cells found in the mixed biofilm. Dual-species biofilm formation by Kp-Ef seemed to increase the number of detached planktonic cells for both species in the medium surrounding the biofilm.

While not the focus of this study, further research will be needed to understand the biological events that determine the specific behavior observed in each case, such as initial adhesion inhibition, population shift, and dispersal promotion. Moreover, spatial distribution of bacteria in dual-species biofilms, determined by confocal laser scanning microscopy in combination with species-specific fluorescent labeling, may provide further confirmation of processes occurring in these dual species biofilms. Overall, the data presented here may add to the understanding of the complex relationships between bacterial species in the context of polymicrobial biofilms formed on urinary catheters.

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## Disclosure statement

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