



# Interactions of microorganisms within a urinary catheter polymicrobial biofilm model

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## “Interactions of microorganisms within a urinary catheter

### polymicrobial biofilm model”

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### Abstract

Biofilms are often polymicrobial in nature, which can impact their behaviour and overall structure, often resulting in an increase in biomass and enhanced antimicrobial resistance. Using plate counts and locked nucleic acid/2'-O-methyl-RNA fluorescence *in situ* hybridization (LNA/2'OMe-FISH), we studied the interactions of four species commonly associated with catheter associated urinary tract infections (CAUTI):

*Enterococcus faecalis*, *Escherichia coli*, *Candida albicans* and *Proteus mirabilis*.

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Eleven combinations of biofilms were grown on silicone coupons placed in 24-well plates for 24 hrs, 37 °C, in artificial urine medium (AUM). Results showed that *P. mirabilis* was the dominant species and was able to inhibit both *E. coli* and *C. albicans* growth. In the absence of *P. mirabilis*, an antagonistic relationship between *E. coli* and *C. albicans* was observed, with the former being dominant. *E. faecalis* growth was not affected in any combination, showing a more mutualistic relationship with the other species. Imaging results correlated with the plate count data and provided visual verification of species undetected using the viable plate count. Moreover, the three bacterial species showed overall good repeatability SD ( $S_r$ ) values (0.1 – 0.54) in all combinations tested, whereas *C. albicans* had higher repeatability  $S_r$  values (0.36-1.18). The study showed the complexity of early-stage interactions in polymicrobial biofilms. These interactions could serve as a starting point when considering targets for preventing or treating CAUTI biofilms containing these species.

### **Keywords**

Biofilm, CAUTI, polymicrobial, plate count, FISH, interactions

### **Introduction**

Biofilms are often polymicrobial in nature, i.e., more than one species of microorganism is present within the biofilm at the same time.(Nadell et al., 2016) This can impact how the biofilm is formed, its properties, behaviour and overall structure, often resulting in an increase in biomass and enhanced capabilities.(Burmølle et al., 2014; Tan et al., 2017) An example of this are multispecies biofilms in catheter associated urinary tract infections (CAUTIs), where microorganisms attach to the catheter surface both inside and outside the lumen causing infections in the urethra and bladder, or catheter blockage.(A. S. Azevedo et

al., 2017; Chua et al., 2017; A. Flores-Mireles et al., 2019) Through metagenomic and metaproteomic analysis we have been able to understand the composition of these multispecies biofilms. The most commonly found species in the consortia are *Escherichia coli* and *Enterobacter spp.* (Choe et al., 2012) Other prevalent microorganisms include *Pseudomonas aeruginosa*, *Klebsiella spp.*, *Proteus spp.*, *Candida spp.* and *Enterococcus spp.* (Dworniczek et al., 2003; A. Flores-Mireles et al., 2019; Norsworthy & Pearson, 2017; Werneburg et al., 2020)

Studies using dual or multi-species models of CAUTIs have shown the impact of these consortia in the growth and behaviour of the biofilm, as well as the challenge they can pose to treatment strategies. (A. S. Azevedo et al., 2016; Cerqueira et al., 2013) Moreover, polymicrobial interactions have been shown to facilitate biofilm growth, boost its matrix production and enhance virulence factors leading to a more severe form of infection. (A. S. Azevedo et al., 2017; Carvalho et al., 2021; A. L. Flores-Mireles et al., 2015) For example, in the study by Tien *et al.*, a dual species *Enterococcus faecalis* – *E. coli* CAUTI model showed that the presence of *E. faecalis* increased the virulence of *E. coli* by facilitating tissue colonisation. (Tien et al., 2017) Additionally, polymicrobial consortia are inherently more resistant to treatment, thanks not only to the increase in biofilm formation which acts as a physical barrier but also the facilitated transfer of resistance genes between microorganisms. (A. S. Azevedo et al., 2016; Beaudoin et al., 2017; Burmolle et al., 2006; Wang et al., 2017) Hence, the study of CAUTIs in multispecies models is important to ensure a better understanding of the biofilms and instruct more effective treatment strategies against them.

Many of the published studies focus on dual species biofilms and rarely go beyond three species. In this paper we examine the early-stage interactions between four

different species of microorganisms commonly associated with CAUTI. For this purpose, we selected *E. coli*, *E. faecalis*, *Candida albicans* and *Proteus mirabilis*. As these species have not been studied together in a CAUTI biofilm model before, we investigated how the behaviour and interactions change when the number of species in a biofilm is increased.

Due to its high prevalence in CAUTI, *E. coli* was chosen to be present in all our multispecies biofilms. Eleven different biofilm combinations were studied using the traditional plate count method as well as locked nucleic acid/2'-O-methyl-RNA fluorescence *in situ* hybridization (LNA/2'Ome-FISH) in combination with confocal laser scanning microscopy (CLSM) to gain more insight into their spatial organisation and 3D structures. Moreover, the issue of repeatability and reproducibility of biofilm data is very important in the field (N. F. Azevedo et al., 2021). Reproducibility refers to the laboratory-to-laboratory variability of a method, while repeatability refers to its within laboratory variability. (Hamilton, 2010) A method must demonstrate acceptable repeatability before it is tested in a multi-laboratory study to assess reproducibility, hence in this study we evaluated the repeatability of our plate count method for each species in each combination.

## Materials and Methods

The descriptions provided in this materials and methods section complies with the recently published guideline on reporting spectrophotometric and fluorometric methods to assess biofilms in microplates. (Allkja et al., 2020)

### Biofilm formation

#### *Culture maintenance and inoculum preparation*

*E. coli* CECT 434 and *E. faecalis* CECT 184 were streak plated from -80 °C glycerol stocks onto Tryptic soy agar (TSA) [Merck, 1.00550 (Broth) and VWR Chemicals

USBIA0950 (Agar)] and grown overnight at  $37\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ . *P. mirabilis* SGSC 3360 was streak plated from  $-80\text{ }^{\circ}\text{C}$  glycerol stocks onto CLED agar [VWR Chemicals, 84668] and grown overnight at  $37\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ . *C. albicans* SC5314 was streak plated from  $-80\text{ }^{\circ}\text{C}$  glycerol stocks onto Seaboard's Dextrose Agar (SDA) [VWR Chemicals, 84685] and grown overnight at  $37\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ .

One or two colonies from each plate were transferred into 15 mL Tryptic Soy Broth (TSB) [Merck, 1.00550] for the bacteria or 15 mL Yeast-extract peptone dextrose (YPD) [Edge BD Difco™, 242820] for the *Candida*, and incubated at  $37 \pm 2\text{ }^{\circ}\text{C}$ , 120 rpm, overnight. An aliquot was sub-inoculated in the respective fresh media at  $37 \pm 2\text{ }^{\circ}\text{C}$ , 120 rpm until exponential growth phase was achieved [OD = 0.100 (620 nm) or  $7.5 \pm 0.5\text{ Log CFU/mL}$  for the bacteria], [OD = 1.400 (620 nm) or  $7.5 \pm 0.5\text{ Log CFU/mL}$  for *Candida*]. The inocula were centrifuged at 3000 g for 10 mins and resuspended in artificial urine medium (AUM) [According to the recipe by (Brooks & Keevil, 1997)] twice to ensure the previous media was removed. The resuspended inocula were diluted in artificial urine medium (AUM) to a concentration of  $5.5 \pm 0.5\text{ Log CFU/mL}$ .

#### *Biofilm growth*

For the study, 11 different types of combinations were performed, including single species, dual species, three species and four species biofilms. ID designations for each combination can be found in Table 1.

Silicone coupons, 1x1 cm [Neves & Neves Lda, Portugal] were cleaned and sterilised according to the procedure described by (Allkja & Azevedo, 2021). Briefly, coupons were cleaned for 30 mins with gentle stirring in warm water with detergent, subsequently washed 5x in ultra-pure water, then submerged in 90% ethanol with gentle stirring for 30 mins and finally washed with ultra-pure water and left to air dry.

The coupons were then placed at the bottom of the wells in a flat bottom 24-well tissue culture plate [Orange Scientific, 4430300N] and sterilised in a flow chamber under ultraviolet (UV) light for 30 mins.

The inocula prepared at a concentration of  $5.5 \pm 0.5$  Log CFU/mL were used for biofilm formation. From this, 1.5 mL/well were added to the plate, which was parafilm and incubated at  $37 \pm 2$  °C, no shaking for 24 hrs. In the case of multispecies biofilms, the inocula were mixed in equal parts according to the combinations in Table 1 prior to being added to the plate at a final volume of 1.5 mL/well. Six coupons/combination were prepared, and three technical repeats were performed per combination. An additional coupon per experiment was prepared with AUM only to check for sterility.

### **Plate count method**

#### *Selective media optimisation*

For the mixed biofilms, selective media were created to be able to differentiate the plate counts for each species. In the case of *C. albicans*, 100 µg/mL of ampicillin [AppliChem GmbH, A0839] was incorporated into SDA which prevented bacterial growth for all three species. For *E. faecalis*, Kanamycin [Eurobio Scientific, GABKAN00-6Z] at 50 µg/mL and Amphotericin B [Sigma-Aldrich, A2942] at 10 µg/mL in TSA were used to inhibit all three remaining species. Chromocult® Coliform agar acc.to ISO 9308-1 [Merck, MERC1.10426.0500] was used to separate *E. coli* and *P. mirabilis* into distinct colonies and successfully inhibit *E. faecalis* and *C. albicans* growth.

#### *Single tube method*

The single tube method as described by (Goeres et al., 2019) was adapted to harvest the biofilm from the coupons for each combination. Briefly, following 24-hr growth

the supernatant was removed from the wells and coupons were washed once in 1.5 mL of 0.85% saline (vol/vol). They were then transferred to a falcon tube containing 10 mL of 0.85% saline (vol/vol). During initial testing it was observed that some *C. albicans* biofilm was left behind on the coupon, so 0.01 % Tween®80 (vol/vol) [Sigma-Aldrich, P1754] was added to the 10 mL of 0.85% saline (vol/vol) as a surfactant to ensure complete biofilm removal. The tubes underwent vortex/sonicate cycles of 30 seconds each, 5 steps in total beginning with vortex. The sonicator bath [VWR USC-1700T Ultrasonic cleaner, 142-0101] was degassed prior to use by turning it on for 5 minutes as advised in the STM protocol. The suspensions were then serially diluted and 100 µL was spread plated (2x plates/sample) onto the respective selective media depending on the combination and species. The plates were incubated for 24 hrs at 37 °C. Resulting colonies were counted, averaged across plates, multiplied by the dilution factor  $10^d$ , divided by the spread plate volume (0.1 mL), and multiplied by the total volume (10 mL) divided by the coupon surface area (1 cm<sup>2</sup>). This was then converted to a log density (LD) Log CFU/cm<sup>2</sup> following the formula (1) below:

$$(1) LD = \text{Log CFU/cm}^2 = \log_{10}[(10^d \times (\text{average colonies}) / 0.1 \text{ mL}) \times (10 \text{ mL}/1 \text{ cm}^2)]$$

In the case of no colony growth, a value of 0.5 per plate was assigned at the lowest dilution counted in the previous formula. This resulted in our limit of detection (LOD) value, 1.699 Log CFU/cm<sup>2</sup>. (Allkja et al., 2021)

### **Fluorescence *in situ* hybridisation**

#### *Probe testing*

Four LNA/2'OMe probes [BioPortugal Lda, PT] targeting specific rRNA sequences on each species were used (Table 2). Each probe contained a different fluorochrome attached to it, selected to minimise spectral overlap. The *E. coli* and *E. faecalis* probes

were provided by Andreia S. Azevedo. They were previously used and tested in mixed species cultures by (A. S. Azevedo et al., 2022). The *P. mirabilis* and *C. albicans* probes were designed to match the hybridisation conditions of the existing probes as described in the work by (Teixeira et al., 2021). Probe specificity and channel separation was tested in mixed suspensions containing all four species (Figure S1). The FISH in suspension method was performed according to the protocol described by (Oliveira et al., 2021).

#### *FISH in biofilms*

The FISH in biofilms protocol as described by (Allkja & Azevedo, 2021) was adapted for use in this study. Following 24-hr growth the supernatant was removed from the wells and coupons were washed once in 1.5 mL of 0.85% saline (vol/vol). The biofilm was fixed and permeabilised by drying for 15 mins at 60 °C, followed by 15-min immersion in 4 % (vol/vol) paraformaldehyde [Acros Organics, 30525-89-4], then 15 mins in 50 % (vol/vol) Ethanol and finally air drying at room temperature. The fixed biofilms were kept at +4 °C until hybridisation. For the hybridisation, working solutions of the probes at 200 nM concentration were prepared in hybridisation buffer (0.5 M of urea [Merck, 1.08487], 50 mM Tris-HCl [Fisher BioReagents, BP153], 0.9 M NaCl [VWR Chemicals, 27808.297]; pH 7.5). In the case of multispecies biofilms, working solutions of probe mixtures at 200 nM according to the specific combination were prepared. 25 µL of the corresponding working solution was added to each coupon, covered with a coverslip, and incubated in a humidified container, at 60 °C for 90 mins, in the dark. The coverslip was removed, and the coupons were immersed in pre-warmed wash buffer (5 mM Tris Base [Fisher BioReagents, BP152], 15 mM NaCl [VWR Chemicals, 27808.297] and 1% Triton X [Panreac, A9778]; pH 10) for 30 mins at hybridisation temperature. A

negative control sample was performed containing only hybridisation buffer with no probe, to check autofluorescence (Figure S2). Moreover, DAPI staining was performed to verify that the probes were penetrating inside of the biofilm (Figure S3). For this, a drop of DAPI [Sigma-Aldrich, 165344] solution at 0.1 mg/mL was added to the coupon for 10 min in the dark, at room temperature following hybridisation, prior to washing. The coupons were left to air dry and transferred onto a 35 mm imaging dish [ibidi®, 81151] containing glycerol based mounting medium [ibidi®, 50001] ready for microscopy. Three biological and three technical replicated were performed per combination. Samples were stored for a maximum of 4 hrs at +4 °C, prior to imaging.

#### *Confocal Laser Scanning Microscopy*

A Laser Scanning Confocal Leica SP5 microscope was used for image acquisition. Biofilms were observed under a 63x/1.30 glycerol immersion objective. Acquisition mode: XYZ; image format: 1024 x 1024 pixels; zoom factor of 1 or 2.5 or 4; pinhole size: 1 Airy unit; line average: 6; Z-step of 1 µm. Five Z-stack images were acquired per coupon, per zoom factor (1 and 2.5). Sequential scanning was used for mixed species samples. Sequence one: ALEXA Fluor™488 (PMT detector, 488 nm laser, emission range 498-553 nm) and ATTO™ 655 (PMT detector, 633 nm laser, emission range 665-741 nm) were detected. Sequence two: ATTO™ 550 (PMT detector, 561 nm laser, emission range 570-601 nm) and Alexa Fluor™ 594 (HyD detector, 561 nm laser, emission range 611-656 nm) were detected. The positions within the coupon were randomly selected to avoid bias. 3D projections and orthogonal views were generated using Imaris Viewer 9.7.2 [Bitplane AG] and LEICA Application Suite X (LASX).

#### **Statistical analysis**

To assess the repeatability of the plate count method for each species per combination, the LD data from all combination for each species was fit to a One-way ANOVA model comparing LD vs combination. This generated the Mean LDs and repeatability standard deviations ( $S_r$ ) for each combination. Additionally, to investigate the variance components affecting the repeatability within each combination, the LD data was fit to a One-way ANOVA model comparing LD vs Day. The ANOVA was followed by Tukeys test (95% CI) to determine statistical equivalence at 97.5% confidence for the mean LDs between experimental days. The equivalency margin of 0.5 logs was chosen based on previous examples in the literature.(Fritz et al., 2015; Nelson et al., 2013) All statistical assessments were performed using Minitab v20® [Minitab 20.3 Statistical Software (2021). State College, PA: Minitab, Inc. (www.minitab.com)]. Graphs were generated using GraphPad Prism v9® [GraphPad Software, LLC Version 9.2.0 (2021)].

## **Results**

### **Species interaction in multispecies CAUTI model**

Understanding how different species interact with one another in polymicrobial biofilms is crucial in our quest to fight the infections caused by them, such as CAUTI. In our study, the first four biofilms are single species ones and serve as a benchmark for the growth of each species i.e., a reference to see how they are affected when grown in combination with other species. The plate count method provided an overall assessment of the abundance of each species within a polymicrobial biofilm. Figure 1 below shows the results of the plate count method for each species in each of the combinations they were present (refer to Table 1 for combination descriptions). A summary of this data can also be found in Table S1 in the supplementary materials file.

Panel A shows that *E. coli* growth is inhibited in every combination where *P. mirabilis* is present. In panels B and C, we can see that *P. mirabilis* and *E. faecalis* growth is not greatly affected in any of the combinations, regardless of the species present. Panel D shows that *C. albicans* growth is affected in all combinations either due to the presence of *E. coli* or *P. mirabilis*, with the greatest effect observed when the latter is present.

Another important aspect of these relationships is the spatial organisation of the various species within each combination. While plate count data can provide information on the composition of the biofilm and overall abundance of each species within the biofilm, it cannot provide any information on the architecture of the biofilm or the spatial arrangement of the different species. Microscopy is an ideal tool to investigate this and, when combined with a plate count assessment, can provide an insight into how the abundance and growth of each species is affected. FISH can be more sensitive than traditional culture methods and has a lower limit of detection.(Almeida et al., 2013; A. S. Azevedo et al., 2019) In our work, the FISH method in combination with CLSM were used on all 11 biofilm combinations (Table 1). Figure 2 shows the spatial organisation of one representative Z-stack collected from each combination. Orthogonal and widefield views of these stacks can be seen in Figure S4. On the top right-hand side of each Z-stack, a pie chart representing the abundance of each species base on the plate count data has been placed. The data represented in the pie charts can be found in Table S1. From the figure, we observe similar trends to other polymicrobial biofilms. An increase in the number of species results in a denser biofilm (Figure 2) and overall higher plate counts (Table S1). Single species biofilms (Figure 2 C1-4) appear to have similar structures, in terms of heights and density, as well as similar plate count numbers (Table S1). For the dual-

species biofilms (Figure 2 C5-7) we observe that the species are well mixed within the biofilm. In the *E. coli* and *E. faecalis* dual biofilm projection (Figure 2, C6) images show that both species are abundant and well mixed in co-aggregated structures typical of a cooperative relationship. In the case of *E. coli* and *P. mirabilis* (Figure 2, C5) and, *E. coli* and *C. albicans* (Figure 2, C7) the projections clearly show one species being dominant and inhibiting the growth of the other (*P. mirabilis* and *E. coli* respectively).

As previously mentioned, the FISH method is more sensitive than plate counts which was reflected in our results as well. In many instances when using LNA/2'OMe FISH, we could observe a greater abundance of cells in the biofilm than the plate counts would suggest e.g., in C5 when detecting dispersed *E. coli* cells (Figure S5). Moreover, in the case of C7, not only does the abundance of *C. albicans* in the images appear greater than what the plate counts suggested, but the species are also co-aggregated which is not typical for an antagonistic relationship. (Elias & Banin, 2012) Similar observations can be made for the three species and four species biofilms as well, where *E. faecalis* seems to grow well despite the presence of other species in the biofilm, while *P. mirabilis* continues to be more dominant and inhibit *E. coli* and *C. albicans* growth. The co-aggregation observed in the dual species biofilms also persists in the triple and four species biofilms (Figure 2, C8-11).

### **Repeatability assessment of the plate count method**

The repeatability of the viable plate count data was evaluated as a means to understand the within laboratory variability. A summary of this analysis can be found in Table 3 below.

From the table we can see that the plate count method shows overall good repeatability for the three bacterial species in all the different combinations tested.

Higher  $S_r$  values were observed for *C. albicans* overall compared to the bacteria, apart from combinations C9 and C11 where its growth was below the LOD, resulting in an  $S_r$  value of 0. Interestingly we observed a difference in hyphal growth in the *C. albicans* biofilms between experimental days (Figure 3) contributing to the poor repeatability observed.

The  $S_r$  value of 0 was also observed for *E. coli* in combinations C8 and C11. For *P. mirabilis* we can see that plate counts are not affected by the combination and the  $S_r$  values are low for all combinations. Whereas, if we look at the other three, we can see that the Log CFU/cm<sup>2</sup> values differ depending on the biofilm combination as in the case of *E. faecalis*, where the repeatability was affected in the four species biofilms. For *C. albicans*, the  $S_r$  values increased with an increase in the number of species within the biofilm, except for cases where the values were below the LOD. This is in contrast with the bacterial species in most combinations, where  $S_r$  values decreased compared to the respective single species biofilm. Another exception to this trend seems to be biofilm C10, where all three species (*E. coli*, *E. faecalis* and *C. albicans*) show increased  $S_r$  values compared to the single species biofilm.

Moreover, as the variability is mostly due to day-to-day sources rather than within plate sources of variability, we evaluated the difference in means from day-to-day for each species in each combination using a Tukeys test. Overwhelmingly the means were statistically significantly equivalent despite the  $S_r$  values. Similarly to the  $S_r$  values, *C. albicans* biofilms were still the exception as they were largely non-equivalent even in the single species biofilm, which once again might be explained by the hyphal growth phenomenon mentioned before.

## Discussion

In recent years, the importance of studying multispecies biofilms has become increasingly evident.(Burmølle et al., 2014) With this study we wanted to investigate the interactions between four different species commonly associated with CAUTI. Eleven different combinations of biofilms were tested, ranging from single to four species. This allowed us to understand how the behaviour and relationships between each species changes when biofilm diversity increases.

In our results *P. mirabilis* appears to be the more dominant species of the four. It inhibits the growth of *E. coli* and potentially *C. albicans* to below the LOD. The inhibitory effect of *P. mirabilis* on *C. albicans* has been previously observed in dual species biofilm models. (Kart et al., 2020) The study by (Hou et al., 2022), observed a similar inhibitory effect in their *in vitro polymicrobial biofilms* of *P. mirabilis* to *E. coli*. Additionally, *In vitro* studies of *P. mirabilis* and urease negative bacteria including *E. coli*, have shown that the latter increase urease production in *P. mirabilis*, thus increasing its pathogenicity. (Armbruster et al., 2017) This could explain the co-aggregation observed in our imaging as the presence of *E. coli* in low numbers is beneficial to *P. mirabilis* (Figure S5). On the other hand, a co-culture study of *P. mirabilis* and *E. coli* *in vivo* showed an increase in biofilm formation by both species.(Alteri et al., 2015) Suggesting that perhaps this relationship might change with time. Considering that *C. albicans* is also urease negative, a similar interaction might be occurring in this scenario as well. Moreover, as this study only looked at 24-hour biofilms, perhaps a longer growth time would show a similar relationship to the *in vivo* study mentioned above.

Another antagonistic interaction observed is the inhibitory effect of *E. coli* on *C. albicans*, which has not been previously studied in a urinary catheter system. *E. faecalis* appears to have a more cooperative relationship with both *E. coli* and *P.*

*mirabilis*. The study by (Gaston et al., 2020) showed that *P. mirabilis* preferentially adheres to *E. faecalis* during biofilm formation, as this relationship facilitates the establishment of a robust biofilm architecture. (Gaston et al., 2020) This co-aggregation was also observed in our microscopy images when these two species were present. A similar relationship has also been observed in studies of dual-species *E. coli* and *E. faecalis* biofilms. (Tien et al., 2017) Interestingly, in our C8 and C11 biofilms where all three of these species are present, the relationship between *E. faecalis* and *P. mirabilis* appears to “win” as *E. coli* growth is still inhibited by *P. mirabilis*. Alternatively, it could also suggest a more passive role in the relationship from *E. faecalis* in its relationship with the other species, where rather than actively contributing to the biofilm, it is simply cohabitating without competing. Additionally, the 3D projections indicate an increase in biofilm density as the number of species within the biofilm increases, which is consistent with polymicrobial biofilm descriptions in the literature. (Røder et al., 2016)

As part of this study, we also decided to evaluate the repeatability of the plate count method. Our results showed good repeatability for all three bacterial species in all combinations. The recorded  $S_r$  values were comparable to the one for the plate count method control data in the recently published interlaboratory study on single species biofilm formation in microplates ( $S_r=0.27$ ). (Allkja et al., 2021) In contrast, the repeatability was not as good for *C. albicans*, with the highest  $S_r$  value being 1.18 for the C10 biofilm. Interestingly, we could see that for single species *C. albicans* biofilms the day-to-day variability might have been in part due to hyphae formation (Figure 3). In days with lower Log CFU/cm<sup>2</sup> values, hyphae formation was increased. In vitro studies of *C. albicans* CAUTI biofilms have shown that growth in urine media promotes hyphae formation. (Bella et al., 2021) In our study, we could observe

that this was not consistent from day-to-day, as in some experimental days there was little to no hyphae presence. From the plate count data, we can also see that the mean Log CFU/cm<sup>2</sup> values for *P. mirabilis* do not differ much between combinations. In fact, there was only a 0.5 log difference between the lowest and highest value obtained. On the other hand, for the other three species, Log CFU/cm<sup>2</sup> values showed more than a 0.5 log difference. Furthermore, for certain combinations the S<sub>r</sub> values were higher e.g., *E. faecalis* in C11 and *E. coli* in C10. Interestingly, while publications delving into polymicrobial biofilms often point out the difficulty in controlling and replicating data when compared to single species biofilms, our repeatability assessment did not always reflect this as S<sub>r</sub> values often were lower in polymicrobial combinations. (Brown et al., 2019; Gabriliska & Rumbaugh, 2015; Røder et al., 2016) For example, in the case of both *E. coli* and *P. mirabilis* only one of the combinations had higher S<sub>r</sub> value compared to the single species biofilm.

Overall, our data showed that the microplate system is a useful tool to study interactions between different species in a simple CAUTI model. However, if we wanted to use our microscopy data more quantitatively we would run into some issues. For example, *C. albicans* appeared on all four channels (Figure S6), and we experienced difficulties distinguishing *E. faecalis* and *P. mirabilis* from one another due to the closeness in spectrum between their respective fluorochromes (Figure S6). These issues were not observed in the experiments performed in suspension (Figure S1) which suggests that the nature of the LNA/2'OMe probes might be a contributing factor. As the probes are negatively charged, their diffusion could be affected by the negatively charged biofilm matrix and lead to binding issues.(A. S. Azevedo et al., 2016; Hobley et al., 2015) To correct these issues, we attempted using both the automatic and manual dye separation tools on the C11 biofilm in the LASX suite. We

were only able to successfully separate the dyes using the manual tool (Figure S7 and S8). This means that we would need to perform the dye separation process manually for each stack, which would be a very long and time-consuming process. Hence, to be able to quantify species specific parameters for the biofilm, a reliable automated dye separation tool, would need to be developed.

## Conclusions

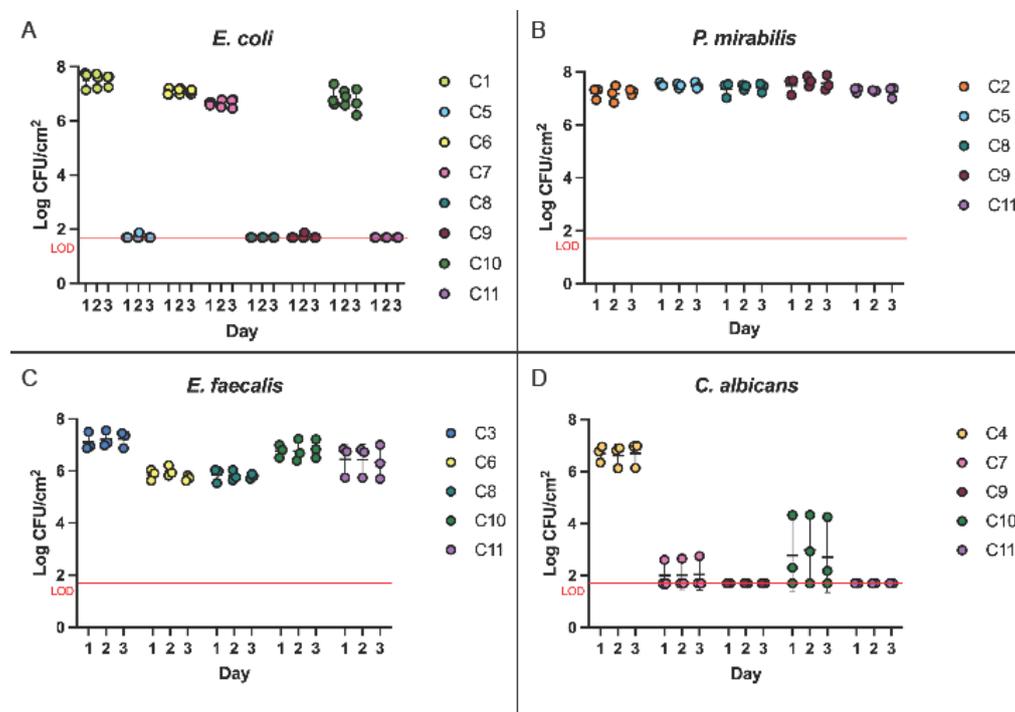
Polymicrobial biofilms are complex and *in vitro* studies of these biofilms are necessary if we want to understand infections such as CAUTI. In this endeavour, the methods used play a crucial role in the information learned about these biofilms and its reliability.. As such, our work demonstrates that a simple device like the microplate can be useful in assessing early interactions between key players in CAUTI such as, *E. coli* and *P. mirabilis*. Moreover, it shows that microscopy is necessary to understand the architecture and spatial organisation of the biofilms. Additionally, the previously unknown interactions unveiled in this work could be a starting point when considering targets for preventing or treating CAUTI biofilms containing these species. Further investigation into the mechanisms of *P. mirabilis* inhibiting *E. coli* or *E. coli* inhibiting *C. albicans* could identify a protein or by-product with antimicrobial activity. Moreover, understanding what gives *P. mirabilis* its edge could provide information on how to stop this species from causing CAUTI biofilms.

## Acknowledgements

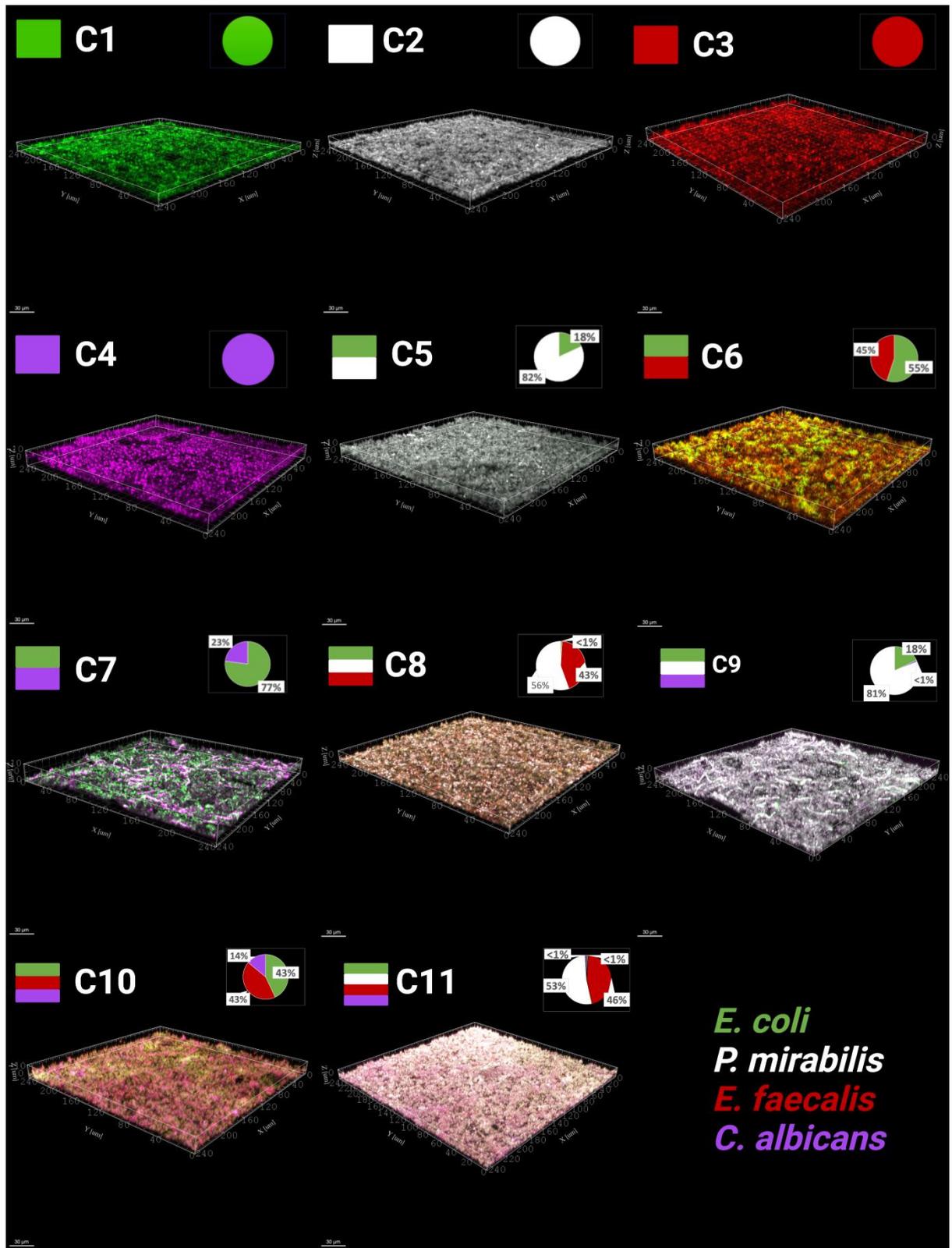
This work was financially supported by: LA/P/0045/2020 (ALiCE), UIDB/00511/2020 and UIDP/00511/2020 (LEPABE), funded by national funds through FCT/MCTES (PIDDAC); project POCI-01-0145-FEDER-030431(CLASInVivo), funded by FEDER funds through COMPETE2020 –

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## Figure Legends

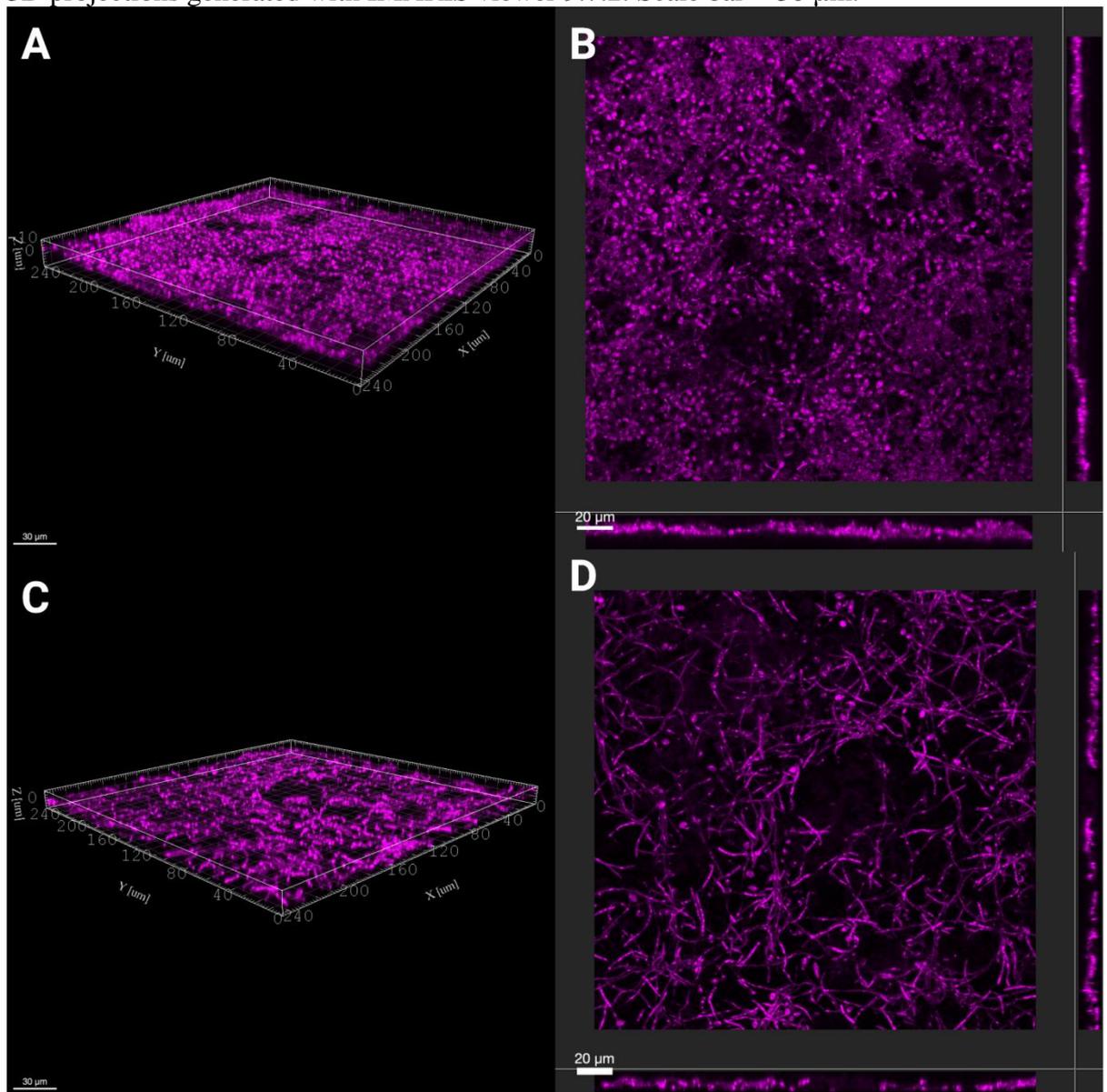


**Figure 1. Plate count method data for each species per combination.** Along the horizontal axis are listed the three experimental days within each combination. Each point in the graph is the log density (Log CFU/cm<sup>2</sup>) on a single coupon. Error bars (Mean LD ± SD) have been plotted for each experimental day. LOD (1.699 Log CFU/cm<sup>2</sup>) represented by the red line. **A.** *E. coli* **B.** *P. mirabilis* **C.** *E. faecalis* **D.** *C. albicans*. Refer to Table 1 for combination definitions.



**Figure 2. Spatial organization of biofilms per combination.** Each species was tagged with a specific colour: *E. coli* (green), *P. mirabilis* (white), *E. faecalis* (red) and *C. albicans* (purple). Coloured panels next to combination ID illustrate the species introduced into the biofilm. Pie charts at the top of each stack represent species abundance based on plate count data. Images were acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective.

Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 1; line average: 6. 3D projections generated with IMARIS viewer 9.7.2. Scale bar = 30  $\mu\text{m}$ .



**Figure 3. Differences in *C. albicans* biofilms between experimental days. A.** Day 1- 3D projection **B.** Day 1 – Orthogonal views. **C.** Day 2- 3D projection **D.** Day 2 – Orthogonal views. For orthogonal views: Central view shows XY plane, bottom view shows XZ plane and Right-side view represents YZ plane. Images were acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 1; line average: 6. Projections generated with IMARIS viewer 9.7.2.

**Competing interests:** The authors declare no competing interests.

**Author contributions:** J.A performed the experiments and drafted the manuscript.

J.A, D.M.G and N.F.A designed the study and reviewed the manuscript. A.S.A designed and provided two of the FISH probes and reviewed the manuscript.

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**Table 1. ID designation (code and color) and description for each combination**

Combination ID	Species
C1 	<i>E. coli</i>
C2 	<i>P. mirabilis</i>
C3 	<i>E. faecalis</i>
C4 	<i>C. albicans</i>
C5 	<i>E. coli and P. mirabilis</i>
C6 	<i>E. coli and E. faecalis</i>

C7 	<i>E. coli</i> and <i>C. albicans</i>
C8 	<i>E. coli</i> and <i>P. mirabilis</i> and <i>E. faecalis</i>
C9 	<i>E. coli</i> and <i>P. mirabilis</i> and <i>C. albicans</i>
C10 	<i>E. coli</i> and <i>E. faecalis</i> and <i>C. albicans</i>
C11 	All 4

Species	Combination	Mean Log	S <sub>r</sub>	Equivalence of	Variance components
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**Table 2. List of LNA/2'OMe probes**

Target	rRNA	Probe LNA/2'OMe (5'-3')	Base pairs	Tag 5'
<i>E. coli</i>	16S	AcGTcAAAtGAgCAaAGg	17	ATTO™ 655
<i>E. faecalis</i>	23S	gTTcTCtGCgTCtAcCtC	18	Alexa Fluor™ 594
<i>C. albicans</i>	18S	cCCGcAtATcTacAa	15	Alexa Fluor™ 488
<i>P. mirabilis</i>	16-23S	cGGaTaTcAtCGGg	14	ATTO™ 550
LNA- lower case designation 2'OMe - upper case designation				

**Table 3. Summary of statistical analysis for the plate count method for each species per combination.** The table shows the main conclusions (Mean Log CFU/cm<sup>2</sup>, S<sub>r</sub>, variance components and equivalency of means testing for day-to-day differences at 97.5% confidence with an equivalency margin of 0.5 logs) for each species by combination.

					Day	Within plate
<i>E. coli</i>	C1	7.517	0.25	Yes	94.74%	5.26%
	C5	1.719	0.06	Yes	25.00%	75.00%
	C6	7.085	0.10	Yes	67.28%	32.72%
	C7	6.653	0.12	Yes	74.22%	25.78%
	C8	1.699	0	Yes	0%	0%
	C9	1.719	0.06	Yes	25.00%	75.00%
	C10	6.816	0.35	No	83.22%	16.78%
	C11	1.699	0	Yes	0%	0%
<i>P. mirabilis</i>	C2	7.209	0.20	Yes	60.57%	39.43%
	C5	7.497	0.09	Yes	73.48%	26.52%
	C8	7.405	0.18	Yes	52.37%	47.63%
	C9	7.570	0.24	Yes	78.78%	21.22%
	C11	7.287	0.12	Yes	24.73%	75.27%
<i>E. faecalis</i>	C3	7.184	0.28	No	83.87%	16.13%
	C6	5.859	0.19	Yes	48.62%	51.38%
	C8	5.859	0.18	Yes	43.38%	56.62%
	C10	6.797	0.31	No	92.32%	7.68%
	C11	6.400	0.54	No	93.42%	6.58%
<i>C. albicans</i>	C4	6.664	0.36	No	94.73%	5.27%
	C7	2.021	0.48	No	99.48%	0.52%
	C9	1.699	0	Yes	0%	0%
	C10	2.823	1.18	No	89.02%	10.98%
	C11	1.699	0	Yes	0%	0%