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In vitro polymicrobial inter-kingdom three-species biofilm model: influence of hyphae on biofilm formation and bacterial physiology

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ABSTRACT

Biofilms are an important medical burden, notably for patients with orthopaedic device-related infections. When polymicrobial, these infections are more lethal and recalcitrant. Inter-kingdom biofilm infections are poorly understood and challenging to treat. Here, an *in vitro* three-species model including *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* was developed, to represent part of the diversity observed in orthopaedic infections or other clinical contexts. The importance of fungal hyphae for biofilm formation and virulence factor expression was explored. Two protocols were set up, allowing, or not, for hyphal formation. Culturable cells and biomass were characterised in both models, and biofilms were imaged in bright-field, confocal and electron microscopes. The expression of genes related to virulence, adhesion, exopolysaccharide synthesis and stress response was analysed in early-stage and mature biofilms. It was found that biofilms enriched in hyphae had larger biomass and showed higher expression levels of genes related to bacterial virulence or exopolysaccharides synthesis.

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Introduction

A biofilm is a consortium of microorganisms embedded in a self- or host-produced matrix that is attached to a biotic or abiotic surface. When self-produced, the components of the matrix are referred to as extracellular polymeric substances, which include mainly, but not exclusively, exopolysaccharides, extracellular DNA, and proteins (Arciola et al. 2018).

Biofilms are considered as a persistent form of infection with a reduced response to antibiotherapies due, among other causes, to immune evasion, reduced penetration or trapping of drugs, enhanced horizontal gene transfer, and dormant phenotypes (Hall and Mah 2017; Crabbé et al. 2019). The complete eradication of biofilms is challenging and the residual cells act as a reservoir of pathogens that may induce a relapse or a chronic infection (Fisher et al. 2017).

Biofilms can be built by a single species, but can also involve different species, including from different kingdoms. Over recent years, the importance of polymicrobial biofilms in human pathologies has been increasingly acknowledged (Røder et al. 2016). Cross-kingdom biofilms, notably composed of bacteria and fungi, are found in denture stomatitis, cystic fibrosis, burn-wound infections, or orthopaedic device-related infections, for example (Nobile and Johnson 2015; Förster et al. 2016). Focussing on orthopaedic infections, it is established that biofilms are polymicrobial in at least 20% of the cases (Xu et al. 2012). *Staphylococcus aureus* and *S. epidermidis* are the most frequently isolated pathogens, but *Cutibacterium acnes,* Enterobacteriaceae, *Pseudomonas aeruginosa* and *Candida* spp., among others, are also involved (Kojic and Darouiche 2004; Larsen et al. 2008).

C. albicans may play a specific role in the development of polymicrobial biofilm infections. This fungus can adopt a dual morphology. It can grow either as a yeast form, i.e. diploid cells that reproduce asexually by gemmation, or as a hyphal form, i.e. branched filaments of cells that have a directional growth. Several *ex vivo* and *in vivo* models have shown that hyphae are the invasive and most pathogenic form of

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Candida spp. Tissue invasion by hyphal *C. albicans* has been demonstrated in mouse tongue and human teeth *ex vivo* (Chupáčová et al. 2018; Dige and Nyvad 2019), or in mouse kidney and tongue or zebrafish *in vivo* (Li et al. 2017; Archambault et al. 2019; Rimachi Hidalgo et al. 2019), as well as in *in vivo* biofilm catheter models (Nobile et al. 2012; Rogiers et al. 2018; Yang et al. 2019). These pieces of evidence lead to the consideration that the presence of hyphae is of major clinical relevance.

In vitro dual-species biofilm models have already provided many insights into the relationship between microorganisms and their response to anti-infective agents. It is well known that bacteria like *S. aureus* or *Escherichia coli* can adhere to hyphae and use them as support to grow (Peters et al. 2012; De Brucker et al. 2015; Kong et al. 2016). This close interaction allows the bacteria to benefit from the protection of secreted fungal products such as exopolysaccharides and confers on them an increased tolerance to antibiotics mediated by the fungal quorum-sensing molecule farnesol (Kong et al. 2017). Also, biofilm-related infections have higher *in vitro* cytotoxicity and higher *in vivo* mortality when polymicrobial than when a single pathogen is present (Peters and Noverr 2013; De Carvalho Dias et al. 2017).

These dual-species models, although necessary, do not represent the variety of possibilities found at the sites of infection. Apart from oral and environmental models, to the best of the authors' knowledge, the only inter-kingdom mixed-species biofilm relevant to orthopaedic device-related infections was published by T. Coenye's group and included *S. aureus*, *Pseudomonas aeruginosa* and *C. albicans* (Kart et al. 2014).

In this article, an original *in vitro* three-species model was developed, including *S. aureus*, *E. coli* and *C. albicans,* which may represent part of the diversity observed in orthopaedic infections as well as in other clinical contexts. Using this model, the importance of the presence of hyphae for biofilm formation and bacterial virulence was explored.

Materials and methods

Strains, culture media and growth conditions

The reference strains *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC47076 and *Candida albicans* ATCC24433 were used in this study.

The microorganisms were stored in Mueller-Hinton broth supplemented with 10% glycerol at −80 °C. For all experiments, pre-cultures were prepared from a frozen aliquot on tryptone soy agar (TSA; BD, Franklin Lake, NJ) or Sabouraud glucose agar (SGA: peptone 10 g l^{-1}, D-glucose 40 g l^{-1}, agar 15 g l^{-1}), for the bacteria and *C. albicans* respectively, and incubated overnight at 37 °C. Aliquots were discarded after thawing.

Inocula were prepared in phosphate buffer saline (PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.5 mM). Two RPMI-based media were used to cultivate the biofilms. The first medium (referred to as RGP) consisted in RPMI-1640 supplemented with L-glutamine (Sigma-Aldrich, St Louis, MO) and buffered at pH 7.4 with KH_2PO_4 50 mM and Na_2HPO_4 74.1 mM. The medium was filter-sterilized and supplemented with 10 g l^{-1} of autoclaved glucose. The second RPMI-1640 medium (referred to as RH), was buffered with 25 mM Hepes (Sigma Aldrich) and not supplemented with glucose. These media were selected based on preliminary experiments aiming at optimising microorganism growth. A series of buffers (9 mM or 28 mM citric acid, 50 mM Tris, 100 mM Tris-malate, or combinations) were tested, together with supplements as foetal bovine serum (FBS: Thermofisher Scientific, Waltham, MA) and bioavailable iron (0.25 mM $(NH_4)_2Fe(SO_4)_2$, 0.06 mM $Fe_4(P_2O_7)_3$ or 0.25 mM $C_6N_6FeK_3$).

The following selective agar media were used for CFU counting: modified mannitol salt agar (MSA: peptone 5 g l^{-1}, NaCl 75 g l^{-1}, D-mannitol 10 g l^{-1}, agar 15 g l^{-1}, amphotericin B 5 mg l^{-1}) for *S. aureus*; selective TSA (TVA: TSA + 5 mg l^{-1} vancomycin + 5 mg l^{-1} amphotericin B) for *E. coli*; selective SGA (S4: SGA + 15 g l^{-1} agar, pH 4.5) for *C. albicans*. Amphotericin B and vancomycin were obtained from Sigma-Aldrich and Mylan (Hoeilaart, Belgium), respectively. Antimicrobials were added after autoclaving when the temperature was below 60 °C.

Biofilm culture in microtiter plates

Biofilms were grown in polystyrene tissue culture plates (96 wells F surface treated; VWR, Radnor, PA). Pre-cultured colonies were suspended in PBS and diluted in RGP or RH. A volume of 200 µl of microbial suspension was added to the wells (culturable surface 1.57 cm^2 $well^{-1}$). Edge wells were filled with sterile media and were used as negative controls. Unless detailed otherwise, inoculum in the wells was adjusted to 1.5×10^7 cfu ml^{-1} for *S. aureus*, 6×10^6 cfu ml^{-1} for *E. coli*, and 2.5×10^6 cfu ml^{-1} for *C. albicans*. Actual inocula were checked by cfu

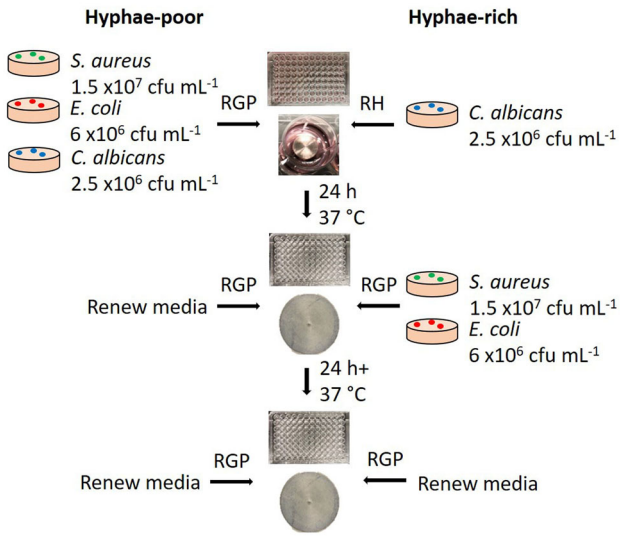

Figure 1. Protocol overview. RGP: RPMI + glucose + phosphate buffer. RH: RPMI + Hepes.

counting. Plates were incubated at 37 °C for 48 to 72 h in darkness. Fresh media were supplied every 24 h (no washing of the biofilm at 24 h and one washing at 48 h). The pH of each medium was estimated over time with pH indicator strips (Merck, Darmstadt, Germany). For the optimisation of growth conditions, non-adherent cells were removed at 90 min of incubation and fresh medium was supplied. Every separate plate was considered as a biological replicate.

For hyphae-poor biofilms, *S. aureus*, *E. coli* and *C. albicans* were co-inoculated for the three-species biofilm, or a combination of one of the bacteria and *C. albicans* for the dual-species biofilm, and *C. albicans* was inoculated alone for the single-species biofilm, all in RGP. For hyphae-rich biofilms, *C. albicans* was inoculated alone and incubated for 24 h in RH. After discarding the medium, RGP inoculated with *S. aureus* and/or *E. coli* for the three- or dual-species biofilms was added. For the single-species *C. albicans* biofilm, fresh RGP was added at 24 h. A schematic overview of the protocols is shown in Figure 1. The optimisation of growth conditions and relative inocula of the three species was performed using the hyphae-poor protocol.

At the end of each incubation period of three-species and dual-species biofilms, the medium was discarded by inversion of the plate. To remove the residual planktonic cells, biofilms were washed by adding 200 µl of PBS along the walls of the well for 10 s. PBS was discarded by inversion and the plates were deposed on absorbent paper. Three-species and dual-species biofilms were washed twice. The medium of single-species *C. albicans* biofilms was discarded by aspiration with a pipette and washed only once instead.

Estimation of culturable cells within biofilms

The number of culturable cells within the biofilms was estimated by colony-forming unit (cfu) counting. Biofilms were detached by mechanical scratching of the surface with an inoculation loop and re-suspended in 200 µl of PBS with vigorous pipetting. The re-suspended biofilms were disaggregated by sonication (Q700; QSonica, Newton, CT) at 60% amplitude 30 s directly in the well. The suspension was recovered, and the content of two wells of each biological replicate was pooled and diluted appropriately. Fifty µl of the same dilution series were transferred to the appropriate selective or non-selective agar. Agar plates were incubated at 37 °C. Colonies on TVA, S4 and SGA plates were counted after overnight incubation, and colonies on MSA were counted after at least 24 h.

Biomass assay

Total biomass was estimated using a protocol adapted from Diaz Iglesias et al. (2019). Briefly, after removal of the medium, biofilms were dried at 60 °C. A volume of 200 µl of crystal violet (Sigma-Aldrich) at 0.5% (V/V, final concentration 115 mg l^{-1}) in water was used to stain the dry biofilms for 10 min at room temperature. Non-bound crystal violet was rinsed with running water. Bound crystal violet was re-solubilized in 200 µl of 66% acetic acid (V/V) (Merck) in water for at least 1 h in darkness and quantified by measuring the absorbance of the solution at 570 nm using a microplate reader (SpectraMax Gemini XS microplate spectrophotometer; Molecular Devices LLC, San José, CA). Blank values were subtracted from at least four sample wells and values were averaged for each biological replicate.

Biofilm culture on titanium coupons

Biofilms were cultured on Ti coupons with a protocol adapted from Poilvache et al. (2020). This material is widely used in the manufacture of orthopaedic protheses, and thus a relevant surface for clinical applications. Briefly, Ti alloy disc coupons (Ti-6Al-4V ELI, diameter 12.7 mm, thickness 3.8 mm; BioSurface Technologies, Bozeman, MT) were placed in 12-well plates with sterile forceps. The inoculum was prepared following the hyphae-poor and hyphae-rich protocols detailed above and 3 ml were added to each

well. Plates were incubated at 37 °C with an orbital shaking of 50 rpm. Every 24 h, the coupons were transferred to fresh medium without washing.

At the end of the experiment, coupons were reconditioned according to a protocol adapted from BioSurface Technologies Co (2019). Briefly, used coupons were immersed in ethanol for at least 24 h, then in 0.1% (V/V) RBS soap and sonicated for at least 10 min. Coupons were rinsed in running water and sonicated consecutively in water until no foam is produced. Coupons were immersed in 2 M HCl for 2 h, rinsed with milliQ water, let dry at 60 °C and autoclaved.

Confocal microscopy of biofilms on Ti coupons

Biofilms on Ti coupons were visualised after labelling *C. albicans* with calcofluor white (Sigma-Aldrich) and the bacteria by fluorescent *in situ* hybridisation (FISH), using a protocol adapted from Nistico et al. (2009). Calcofluor white is a fluorescent lectin that binds to β-polysaccharides, notably β-glucans from the *C. albicans* cell wall. FISH allowed staining specifically of each bacterial species with a fluorescently labelled DNA probe. The oligonucleotides Sta697-FITC and Ent168-Atto550 for *S. aureus* and *E. coli,* respectively, were provided by Metabion (Planegg, Germany) and used as probes (Table S1). They were checked for cross-identities and hairpin formation. Fresh biofilms without washing were fixed with 40 mg l^{-1} of formaldehyde in PBS for 1 h at room temperature, and cells were permeabilized in pre-heated permeabilization reagent (Tris-HCl 20 mM, EDTA 5 mM, lysozyme 1 mg ml^{-1} (Sigma-Aldrich), lysostaphin 0.1 mg ml^{-1} (Sigma-Aldrich), pH 7.5) for 10 min at 37 °C. Biofilms were incubated first with hybridisation buffer (Tris-HCl 20 mM, NaCl 900 mM, SDS 0.1 mg l^{-1}, formamide 20% V/V, pH 7.5) alone for 15 min at 46 °C and then with 0.75 µM of each probe and 0.05 mg ml^{-1} of calcofluor white in the same buffer for 3 h at 46 °C in darkness. Non-bound probes were washed with washing buffer (Tris-HCl 20 mM, EDTA 5 mM, SDS 0.1 mg l^{-1}, NaCl 450 mM, pH 7.5) for 15 min at 46 °C. Excess of liquid was discarded carefully with an absorbent paper and coupons were mounted with DAKO mounting oil (Agilent, Santa Clara, CA) and a glass coverslip. Z-stack pictures of the stained biofilms were taken with an AxioImager.z1-ApoTome microscope (Carl Zeiss, Oberkochen, Germany) through a multi-acquisition from the top to the bottom of the biofilm. Mean of filter-sets (excitation/emission) were blue 365/450 nm, green 460/550 nm and red 535/590 nm. Pictures were analysed and converted to maximal intensity projections (MIP) with AxioVision Rel. 4.8.2.0 (Carl Zeiss).

Scanning electron microscopy

Biofilms grown on Ti coupons were visualised in a scanning electron microscope (SEM) using a protocol adapted from De Brucker et al. (2015). Briefly, biofilms were fixed with 2.5% glutaraldehyde (Sigma) in sodium cacodylate buffer 0.1 M at pH 7.4 (Sigma) for 30 min, washed in PBS and dehydrated by consecutive incubations of 20 min each in 30, 50, 70, 90, and three times 100% ethanol (Merck). After drying, biofilms were coated with Au using a sputtering device (Blazers) and visualised using a FEI XL30-FEG SEM at high-vacuum with a 10 keV voltage.

Gene expression from biofilms

The genetic expression in the biofilms was analysed for *hla*, *clfA*, *hld*, *icaA*, *psmα, fnbB, atl, sigB* and *rsh* in *S. aureus* and *fimA*, *pgaC*, *rpoS* and *relA* in *E. coli*. The primers used are detailed in Table S2. Hyphae-poor and hyphae-rich three- and dual-species biofilms were cultured as detailed above. The samples were recovered at different time-points according to the maturity of the biofilm. Hyphae-poor biofilms were recovered at early-stages (time-point 4 h) and late-stages (time-points 28 and 48 h). Hyphae-rich biofilms were recovered at early-stages (time-point 28 h, 4 h after inoculum of bacteria) and late-stages (time-point 48 h). Biofilms were recovered with five successive thorough washes with 200 µl of recovery buffer (Tris-HCl 10 mM, EDTA 1 mM in DEPC-treated water (Thermofisher Scientific) at pH 8.0). All washes from the same condition were pooled on the same tube and were centrifuged 3,000 g for 10 min. The pellet was suspended in 1 ml of recovery buffer, transferred to a new tube and centrifuged at 10,000 g for 2 min. The pellet was incubated with 100 µl of 3 mg ml^{-1} lysozyme in recovery buffer for 30 min at room temperature. When *S. aureus* was present in the sample, 1 mg ml^{-1} lysostaphin (Sigma Aldrich) was added with lysozyme. RNA was extracted using the Invitrap Spin Cell RNA mini kit (Invitek Molecular, Berlin, Germany) following the manufacturer's instructions. Residual DNA was eliminated with the TURBO DNA-free kit (Thermofisher Scientific) following the manufacturer's instructions. cDNA was synthesised using the First Strand cDNA Synthesis kit for RT-PCR (Roche, Basel, Switzerland) following the manufacturer's instructions. In parallel, samples were

prepared from planktonic cultures of pure *S. aureus* or *E. coli* and mixed *S. aureus* and/or *E. coli* and *C. albicans*, collected at 4 h in an exponential growth phase. The relative quantification of the cDNA was performed with a CFX96 Touch Real-Time PCR (95 °C 3 min, 95 °C 10 s, 60 °C 30 s, 40 cycles; Bio-Rad, Hercules, CA), using 5 µl of sample and 20 µl of PCR mix (Sso Advanced Universal SYBR Green Supermix 12.5 µl (Bio-Rad), DEPC water 4 µl, primer Fw 2 µl, primer Rv 2 µl per reaction).

Data and statistical analyses

Calculations, statistical analyses and plots were performed using Microsoft Excel and GraphPad Prism 8.4.3 (GraphPad Software, San Diego, CA). qPCR results were recorded with Bio-Rad CFX Maestro 1.1 (Bio-Rad) and analysed according to the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen 2001).

Results

Buffer system and inoculum optimisation

The buffer system and the inoculum were optimised for the three-species biofilm with reference strains of *S. aureus*, *E. coli* and *C. albicans*. The aim was to set up a protocol that allowed culturing a biofilm with high biomass and containing more bacteria than fungi, with no overgrowth of one of the bacterial species over the others, and remaining stable for at least 72 h. The development of the model was performed first with all the species added at the same time.

Several buffer systems were tested in RPMI + 1% glucose and the results are represented in Figure S1. Biofilms cultured with either phosphate or Hepes buffers showed the more abundant biomass at 48 h. Yet only the phosphate buffer at the highest concentration tested (50 mM $H_2PO_4^-$/74.1 mM HPO_4^{2-}) was able to maintain the pH above 6 from 24 to 72 h and was thus selected for further experiments. Other discarded growth conditions are detailed in the supplemental information and Figure S2.

Several combinations of the relative initial inocula were tested for the three species. Biomass reached similar levels at 48 h (Figure S3), with absorbance ranging between 0.75 to 1.25 AU cm^{-2}. Conversely, changing the inoculum and the relative proportions of the three species allowed modulation of the resulting culturable cells in the biofilms (Figure S4). Finally, an inoculum of $15{:}6{:}2.5 \times 10^6$ (*S.aureus:E.coli:C.albicans*; Figure S4J) was selected as it showed the most stable culturable cell values up to 48 h.

Further details of the optimisation of the growth conditions can be found in the supplemental information.

Imaging of biofilm and hyphal morphogenesis

The resulting three-species biofilm, as well as the corresponding dual-species bacteria-fungi biofilm and the single-species *C. albicans* biofilm, were imaged in bright-field microscopy. In a routine approach, the biofilms were imaged on the bottom of the 96-wells plates after staining with crystal violet (Figure 2, left panels). The pictures were taken at centred regions, where a rather uniform coverage of the surface was observed, but regions closer to the walls showed higher biomass. Differentiating between the bacteria was not possible but *C. albicans* appeared mostly in yeast morphology. These biofilms are therefore considered as hyphae-poor.

As hyphae are considered the pathogenic form of *C. albicans*, the protocol was modified to increase their abundance. To this effect, *C. albicans* was cultured 24 h in RH before inoculating the bacteria. The increased hyphal morphogenesis was confirmed visually (Figure 2, right panels). Biofilms prepared with this protocol are referred to as hyphae-rich.

The subsequent dual- and single-species biofilms were also visualised. The ultrastructural morphology of the *S.aureus:C.albicans* dual-species hyphae-rich biofilm was similar to that of the three-species hyphae-rich biofilm, with increased abundance of hyphae in comparison with the *S.aureus:C.albicans* hyphae-poor model. The *E.coli:C.albicans* dual-species hyphae-rich biofilm showed a similar coverage of the surface compared with the *S.aureus:C.albicans* hyphae-rich model, as well as an increased abundance of hyphae compared with the *E.coli:C.albicans* hyphae-poor model. Single-species *C. albicans* biofilm showed a very large abundance of hyphae in the hyphae-rich biofilm.

Characterization of hyphae-poor and hyphae-rich models

The culturable cells and the biomass of the resulting biofilms were estimated and compared in the hyphae-rich and the hyphae-poor models. The results of the three-species biofilms are represented in Figure 3 and the values of all the biofilms are detailed in Table 1. In the three-species biofilms, there was no significant difference in the culturable cells of any of the microorganism at 48 or 72 h, but biomass was significantly

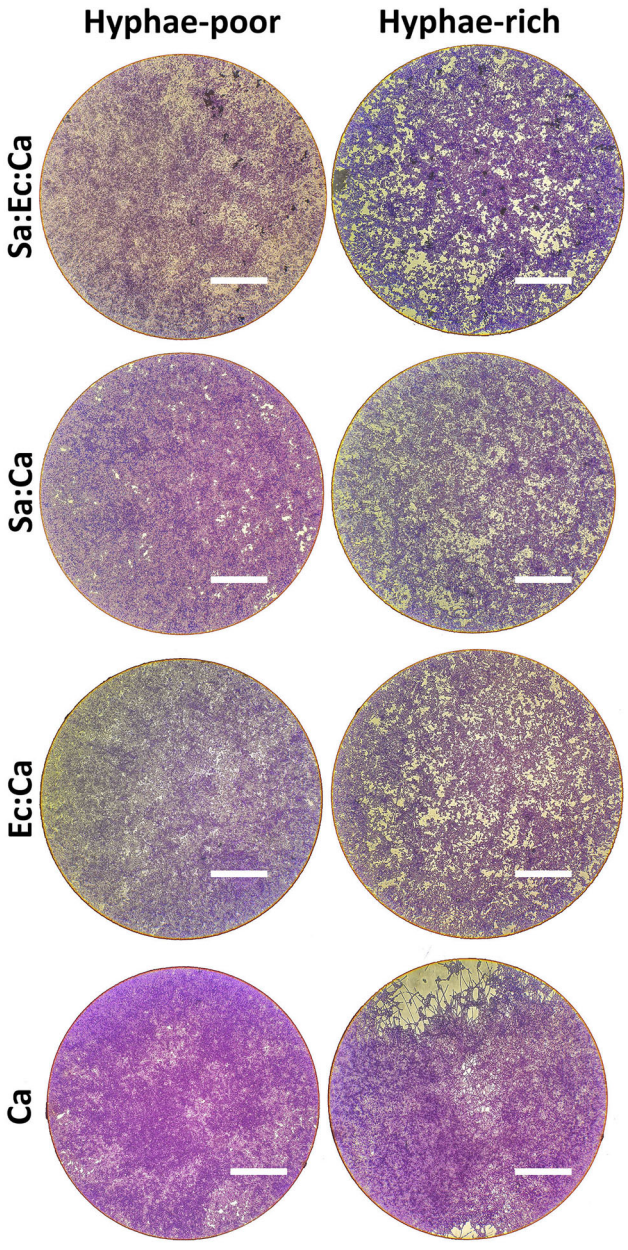

Figure 2. Bright-field microscopy of 48 h biofilms on 96-wells plates, stained with crystal violet (0.5% 10 min), cultured under the two different protocols. Magnification 20x. Bars 20 µm. Sa: *S. aureus*. Ec: *E. coli*. Ca: *C. albicans*.

higher in the hyphae-rich model. Conversely, *S.aureus:C.albicans* dual-species hyphae-rich biofilm showed significantly lower values than the hyphae-poor for both culturable cells and biomass at 48 and 72 h, while *E.coli:C.albicans* biofilms showed no significant difference between the two protocols. Lastly, *C. albicans* single-species hyphae-rich biofilm showed a significant increase in biomass at both times and of culturable cells at 72 h as compared with the corresponding hyphae-poor model.

Spatial organisation

To visualise the organisation of the different microorganisms within the biofilm and confirm the enrichment in hyphae, they were imaged on Ti coupons after staining with FISH and calcofluor white (Figure 4A). The blue channel for calcofluor white fluorescence alone is shown in Figure 4B as a greyscale image.

For the hyphae-poor three-species biofilm, *C. albicans* was heterogeneously spread forming clusters with a vast majority of yeast forms (Figure 4B). *E. coli* appeared to be more abundant than *S. aureus*, but this could be an artefact due to the increased difficulty of permeabilizing *S. aureus* cells. No preferential interaction between the bacteria and *C. albicans* was observed. Bacteria rather homogenously occupied the spaces among the fungal clusters.

For the hyphae-rich biofilms, the abundance of hyphae was much larger in all the biofilms (Figure 4B). *C. albicans* distribution was more disperse and bacteria formed clusters close or among the hyphae (Figure 4A). Again, in the three-species biofilms, *E. coli* seemed to be more abundant than *S. aureus*, but less abundant than in the three-species hyphae-poor biofilm.

Scanning electron microscopy

The differences between the hyphae-poor and the hyphae-rich biofilms were confirmed after visualisation of the three-species biofilms using SEM (Figure 5). The hyphae-poor protocol formed thin biofilms with a low abundance of matrix. *C. albicans* appeared mainly in yeast forms and bacteria were essentially clustered around the rare hyphae. On the contrary, the hyphae-rich protocol formed thick biofilms, consisting mainly of interwoven hyphae. The matrix was found mainly close to the hyphae and encapsulated bacteria from both species forming clusters.

Gene expression

The expression of bacterial genes was compared among the three- and dual-species mixed planktonic cultures and biofilms. The genes selected were associated with virulence, adherence, exopolysaccharide synthesis, and environmental stress. All the primers were specific for the corresponding bacteria with the conditions used (Table S3). The results of the expression of the studied genes are shown in Figures 6, S5 and S6 (for the three-species biofilms, the dual-species *S.aureus:C.albicans* biofilms and the 2-species *E.coli:C.albicans* biofilms, respectively). The multiple comparison tests are detailed in Table S4.

Considering the three-species biofilms first, the expression of the genes encoding the virulence factors

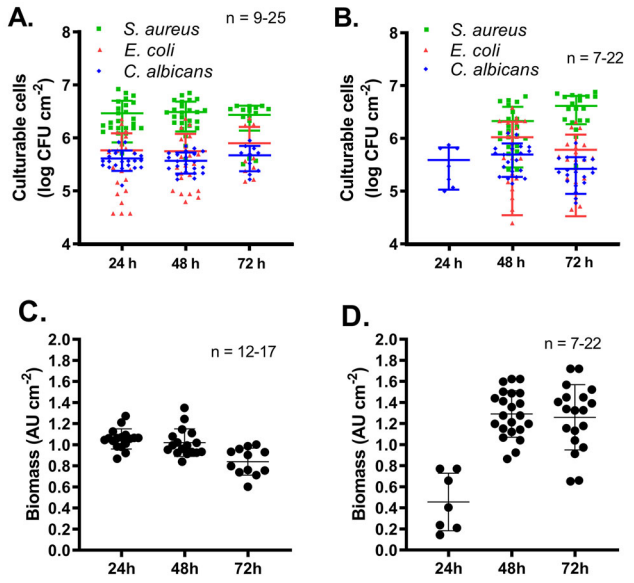

Figure 3. Culturable cells (A, B) and biomass (C, D) of the resulting 3-species *S. aureus: E. coli*: *C. albicans* biofilm at 24, 48 and 72 h, with an inoculum of 15:6:2.5 x10^6 cfu ml^{-1}. A, C: hyphae-poor model. B, D: hyphae-rich model.

Table 1. Culturable cells and biomass of biofilms including at least *C. albicans*, cultured in RPMI + 1 % glucose + 50/74 mM $H_2PO_4^-$/HPO_4^{2-} with the two different protocols.

			Culturable cells (log cfu cm^{-2})		Biomass (AU cm^{-2})	
Time	Model		Hyphae-poor	Hyphae-rich	Hyphae-poor	Hyphae-rich
48 h	3-sp	Sa	6.4 ± 0.3	6.3 ± 0.5	1.02 ± 0.13*	1.28 ± 0.22*
		Ec	5.6 ± 0.6	5.5 ± 0.5		
		Ca	5.5 ± 0.2	5.7 ± 0.6		
	Sa:Ca	Sa	6.7 ± 0.2*	6.2 ± 0.2*	1.73 ± 0.14*	1.26 ± 0.18*
		Ca	6.2 ± 0.1*	5.7 ± 0.5*		
	Ec:Ca	Ec	5.9 ± 0.6	6.4 ± 0.9	0.95 ± 0.2	0.75 ± 0.24
		Ca	5.7 ± 0.1	5.5 ± 0.4		
	1-sp	Ca	5.7 ± 0.3	5.8 ± 0.6	0.49 ± 0.19*	1.23 ± 0.36*
72 h	3-sp	Sa	6.4 ± 0.3	6.6 ± 0.4	0.84 ± 0.13*	1.25 ± 0.3*
		Ec	6 ± 0.9	5.6 ± 0.4		
		Ca	5.6 ± 0.3	5.5 ± 0.4		
	Sa:Ca	Sa	7 ± 0.2*	6.4 ± 0.2*	1.79 ± 0.12*	1.21 ± 0.18*
		Ca	6.2 ± 0.3*	5.5 ± 0.3*		
	Ec:Ca	Ec	5.7 ± 0.3	6.4 ± 0.4	1.23 ± 0.38	0.82 ± 0.39
		Ca	5.4 ± 0.2	5.6 ± 0.3		
	1-sp	Ca	5 ± 0.2*	5.8 ± 0.3*	0.36 ± 0.27*	1.57 ± 0.26*

Sa, *S. aureus*; Ec, *E. coli*; Ca, *C. albicans*; *, significant difference (p-value < 0.05) between hyphae-poor and hyphae-rich protocols, non-parametric ANOVA. N = 7–22.

haemolysin delta (*hld*), haemolysin alpha (*hla*) and clumping factor A (*clfA*) by *S. aureus* was up-regulated for incubation times longer than 28 h (Figure 6A–C). In 48 h biofilms, all three genes were expressed to higher levels in hyphae-rich biofilms. The expression patterns of the membrane adhesion proteins tested, fibronectin-binding protein B (*fnbB*) and autolysin (*atl*), were different (Figure 6D,E). *fnbB* was up-regulated 4 h after the bacterial inoculation, especially in hyphae-rich biofilms at 28 h, and its expression was greatly reduced afterwards. On the other hand, *atl* expression was increased from 28 to 48 h, especially in the hyphae-rich biofilm. PNAG synthase (*icaA*), responsible for exopolysaccharide synthesis, showed an expression pattern similar to that of the virulence genes, with increased expression over time reaching higher levels in hyphae-rich vs hyphae-poor biofilms (Figure 6F). Phenol-soluble modulin alpha (*psmα*), involved in biofilm dispersion, was substantially down-regulated at 4 h in the three species hyphae-poor model (to a level similar to those measured in the mixed planktonic cultures), as well as at 28 h in the hyphae-rich models (Figure 6G). But its expression increased over time after the inoculation of bacteria. The negative and positive stress response regulators RNA polymerase sigma beta cofactor (*sigB*) and pyrophosphokinase (*rsh*) showed a stable expression, with levels remaining close (less than 2 times change) to those measured in the corresponding planktonic cultures (Figure 6H,I), except for *sigB* which was 5.0 ± 0.1 times down-regulated in the hyphae-poor biofilm. The expression of these genes by *S. aureus* in the dual-species *S.aureus:C.albicans* biofilms and the comparison between hyphae-poor and hyphae-rich biofilms were similar to those observed in the three-species biofilms (Figure S5).

In *E. coli*, type-1 fimbriae protein A (*fimA*) and PNAG synthase (*pgaC*) were up-regulated and stable at the different times analysed (Figure 6J,K). RNA polymerase subunit S (*rpoS*) and pyrophosphokinase (*relA*), both positive stress regulators, were upregulated in the biofilms as well as in the planktonic cultures, especially for *rpoS*. This high expression was maintained over time in all biofilm models (Figure 6L,M).

The expression profile of *E. coli* in the dual-species *E.coli:C.albicans* biofilms was similar except for the expression of *pgaC*, which showed an increase from 4 to 24 h after bacterial inoculation, from 1.2 ± 0.1 to 8.4 ± 0.6 fold-change for hyphae-poor biofilms and from 1.9 ± 0.4 to 23.1 ± 1.9 fold-change for hyphae-rich biofilms (comparing time-points 4 to 28 h for hyphae-poor biofilms and time-points 28 to 48 h for hyphae-rich biofilms, Figure S6).

Discussion

This article explores the formation of *in vitro* inter-kingdom biofilms including the fungus *C. albicans* and two bacteria, *S. aureus* and *E. coli*. A comparison between two protocols that allows biofilms rich and poor in hyphae to be obtained was performed to study the influence of these structures on biofilm formation.

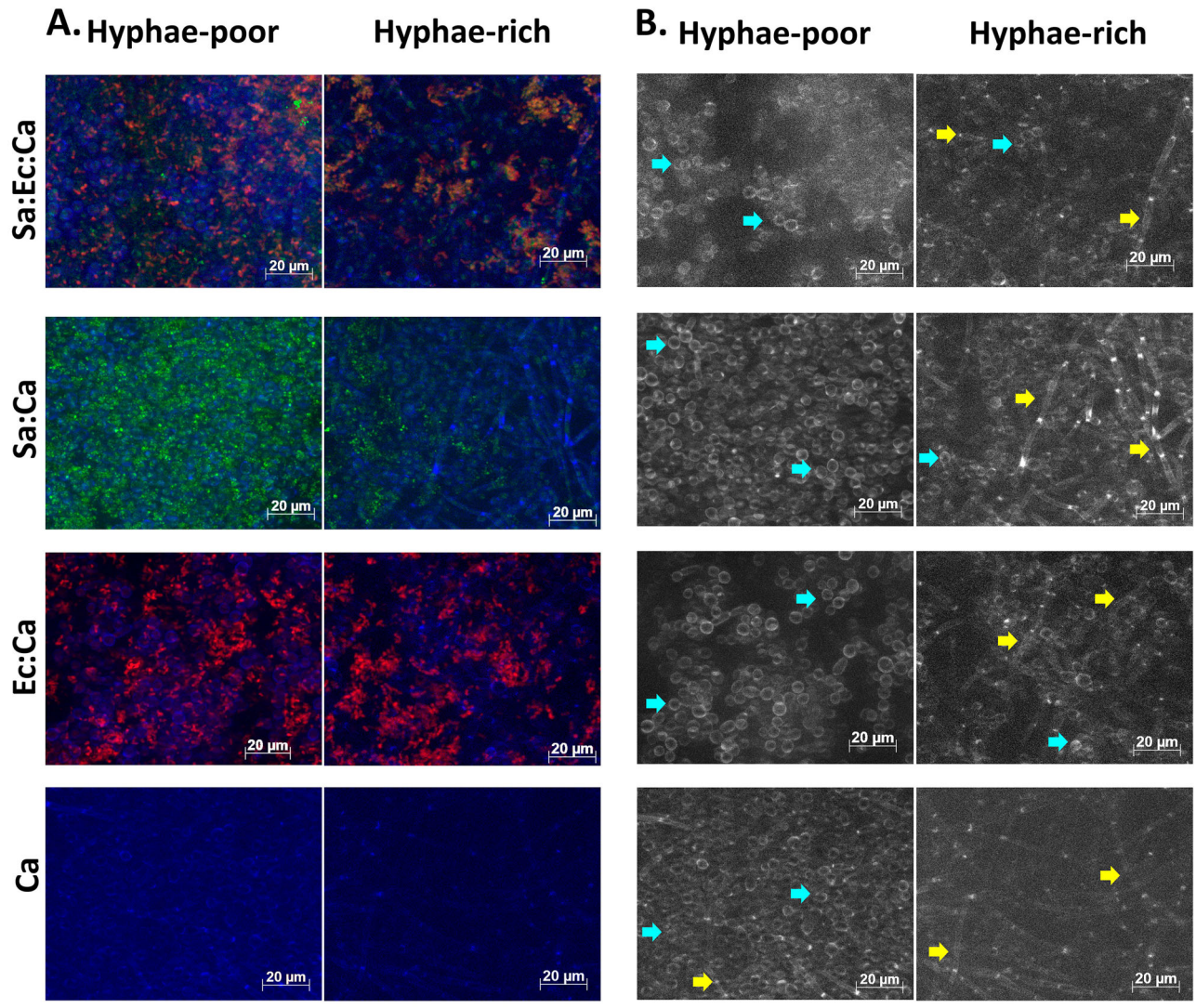

Figure 4. Maximal intensity projections of fluorescence microscopy imaging of 48 h biofilms on Ti coupons after FISH and calcofluor white staining, cultured with the protocols for hyphae-poor and hyphae-rich biofilms. Bars: 20 µm. A: merging of the three channels green (*S. aureus*, Sa), red (*E. coli*, Ec) and blue (*C. albicans*, Ca). B: channel blue on greyscale. Light blue arrows: yeast cells. Yellow arrows: hyphal cells.

A critical parameter in biofilm formation is the choice of the culture medium. In this work, an RPMI-based medium was selected according to most previous *in vitro* inter-kingdom dual-species biofilms, which used a wide variety of growth conditions and strains (Peters et al. 2010; Pammi et al. 2013; De Brucker et al. 2015; Kong et al. 2016; De Carvalho Dias et al. 2017). This medium, recommended by EUCAST for antifungal susceptibility testing in liquid culture, is the more appropriate to ensure fungal growth (Arendrup et al. 2017). In addition, maintaining a pH higher than 6 and supplementing the medium with glucose contributed to boosting and maintaining a stable biomass over time, in accordance with previous work.

Different parameters are frequently used to characterise and quantify biofilm formation. In this article, two quantitative methods were used. A quantification of the culturable cells was performed to estimate the number of viable cells in the biofilms. In parallel, a quantification of the total biomass was performed to analyse the total amount of biofilm including cells and matrix. Both methods are complementary, and together with the qualitative microscopy analysis, they allow the critical differences between biofilms to be studied. In this article, it is relevant and important to use both quantitative methods due to the difference in cell volumes and the nature of the synthesised matrix between the different microorganisms, and notably between the fungus and the bacteria.

Regarding the viable cell counts, a very wide variability is reported in the literature, depending on the model used for growing biofilms. Therefore, the present data were compared only with studies giving

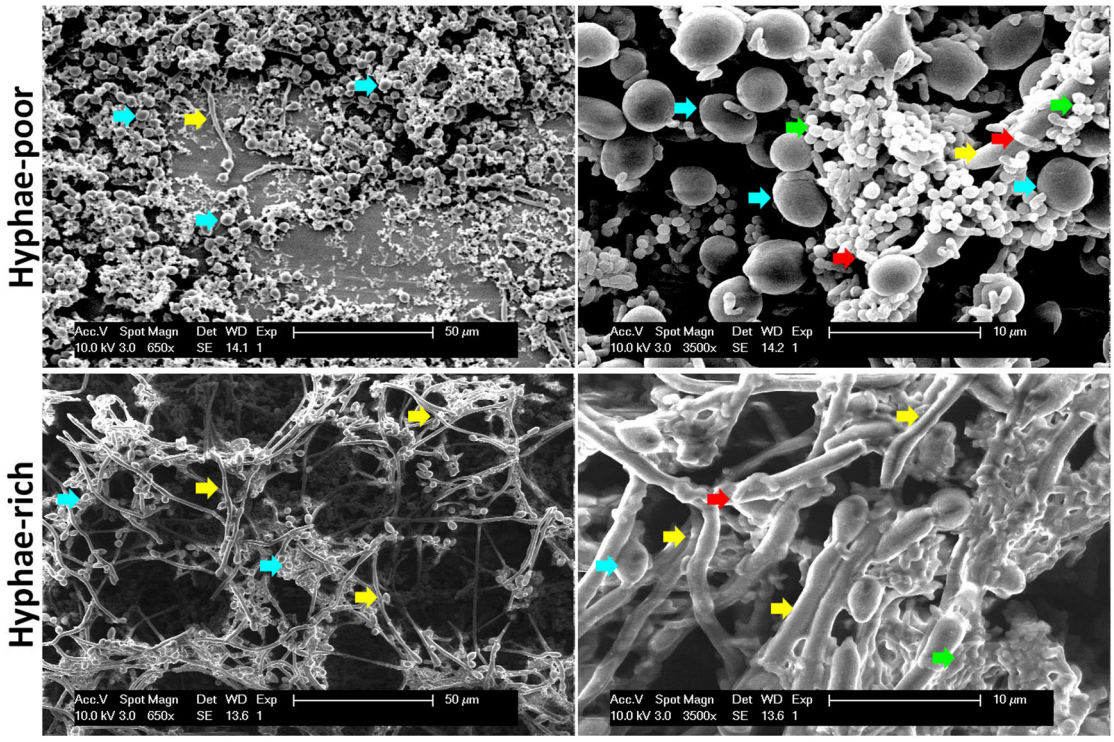

Figure 5. SEM images of 48 h biofilms on Ti coupons cultured under the protocols for hyphae-poor (top) and hyphae-rich (bottom) biofilms. The images were taken at magnifications 650x (left, bar 50 µm) and 3,500x (right, bar 10 µm). Light blue arrows: yeast cells. Yellow arrows: hyphal cells. Red arrows: *E. coli*. Green arrows: *S. aureus*.

enough information to estimate the culturable cells per culturable surface (Table S5). In the authors' hands, the survival and proliferation of bacteria in the biofilm was highly dependent on the relative ratio of their initial inoculum rather than of their absolute initial value. Modifying the *C. albicans* inoculum also influenced the relative abundance of the bacteria but did not markedly change that of the fungus in the biofilm. This differs from what has been reported in an *E.coli:C.albicans* biofilm (grown in RPMI buffered with MOPS), where the initial inoculum of each of these species defined its final abundance but did not influence that of the other species (De Brucker et al. 2015). In this dual-species model, *E. coli* density was around 1 log cfu cm^{-2} higher than in the present dual or three species biofilms using equivalent inocula. Conversely, the abundance of *S. aureus* in the present study (around 2×10^6 cfu cm^{-2}) is strictly in accordance with that described in a three-species biofilm of *S.aureus:P.aeruginosa:C.albicans* grown in BHI + 5% BSA (Kart et al. 2014) and a dual-species biofilm of *S.aureus:C.albicans* grown in RPMI buffered with Hepes (Kong et al. 2016). Other authors, however, report 1 and 2 log cfu cm^{-2} higher abundances, respectively, in dual-species biofilms of *S.aureus:C.albicans* grown in 50% BSA (Harriott and Noverr 2010) or RPMI (Zago et al. 2015). Regarding *C. albicans*, most studies report a similar number of culturable cells in single-species biofilms as found here, but a reduction in its abundance in dual- and three-species biofilms (Kart et al. 2014; Zago et al. 2015), opposed to what the present authors observed. Of note, Kart et al. (2014) had the well-known competitor *P. aeruginosa* in the biofilm, which may explain this difference.

When comparing the hyphae-poor and -rich models, no major differences in cell counts were observed, but were in biomass, which was more important in hyphae-rich models for mono-species and three-species biofilms, but lower in dual-species biofilms. This is in accordance with the results of Kong et al. (2016) who reported a sharp increase in biomass when the fungus was co-cultured with *S. aureus*. Absolute values of biomass are hardly comparable with published data as the experimental protocols used for crystal violet staining differ among publications and greatly influence the results.

The microscopy images, using both confocal and scanning electron microscopy, show the differences between the structures of the hyphae-poor and the hyphae-rich biofilms. The enrichment in hyphae permits the formation of thicker biofilms forming a net-like structure, in accordance to what is seen in *in vivo C. albicans* biofilms models (Lazzell et al. 2009;

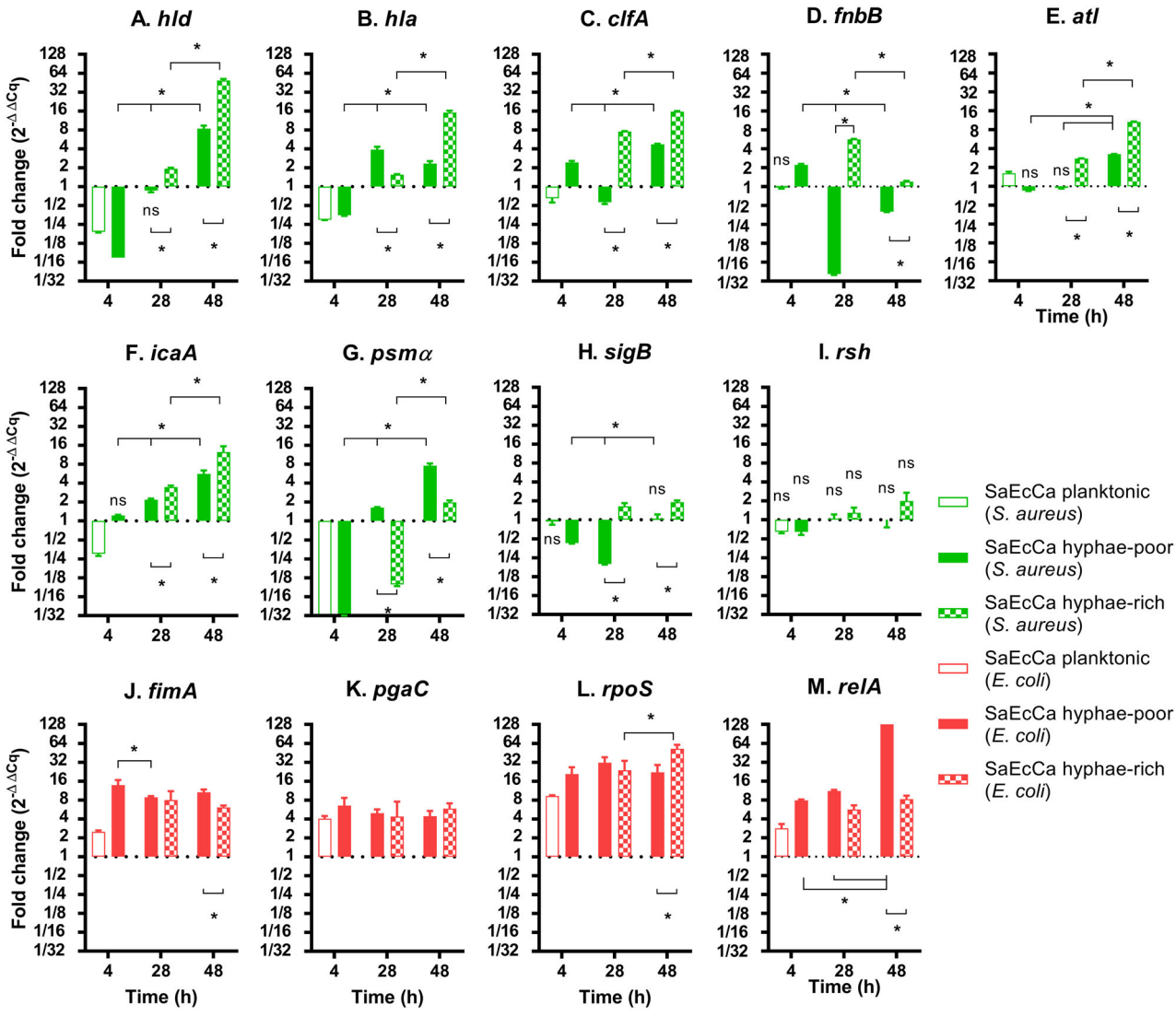

Figure 6. Expression of genes of interest in the three-species biofilms cultured under the hyphae-poor and hyphae-rich protocols. Sa: *S. aureus*. Ec: *E. coli*. Ca: *C. albicans*. Housekeeping genes: *gmk* for *S. aureus* and *gapA* for *E. coli*. The results are expressed as fold change relative to a pure planktonic culture in exponential growth (y-axis) at different time points (x-axis). n = 3. ns: no significant difference from pure planktonic culture. *: significant difference between the time-points or protocols. One-way ANOVA, Tukey post-test.

Nobile et al. 2012). The hyphae-rich biofilm also shows a higher production of matrix, especially close to the hyphae. This matrix allows the formation of clusters of bacteria around the hyphae. Although the experimental design used does not permit discernment of the origin of the matrix, other articles have reported the coating of *S. aureus* or *E. coli* cells by a *C. albicans* matrix in dual-species *innvitro* models (De Brucker et al. 2015; Kong et al. 2016).

The transcriptomic analysis revealed only a few but important differences in gene expression between the hyphae-poor and the hyphae-rich models, essentially in *S. aureus*. The expression of *hld*, *hla* and *clfA*, which encode virulence factors in *S. aureus*, increased over time, and to higher levels in hyphae-rich than in hyphae-poor models. This suggests that mature biofilms synthesise more virulence-related molecules than planktonic bacteria and that their synthesis is up-regulated in the presence of hyphae. *hla* and *hld* are both induced by the *agr* operon, which is stimulated by bacterial cell densities and repressed by environmental stress (Bronesky et al. 2016). Of interest, it is also induced by *C. albicans* (Todd et al. 2019). In addition, *hld,* also known as RNAIII, further stimulates the expression of other virulence factors, including *hla* (Tan et al. 2018). ClfA is a cell wall anchored protein that promotes bacterial adhesion to the plasma protein fibrinogen, facilitating thereby the

colonisation of protein-coated biomaterials. It is detected in a large proportion of strains producing high amounts of biofilm (Zmantar et al. 2008). To the best of the authors' knowledge, its regulation is not fully understood but it is independent of the *agr* operon (Xue et al. 2012). The expression of FnbB, another adhesion protein that recognises fibronectin, was increased in the hyphae-rich model at 28 h. This is coherent with a previous observation that the adherence of *S. aureus* to *C. albicans* hyphae is impaired in an *fnbB* deletion mutant (Schlecht et al. 2015). Lastly, the expression of *icaA* was up-regulated over time and to higher levels in hyphae-rich models. This indicates an increase in the synthesis of PNAG to build up the matrix at later stages of the biofilm, which is further stimulated in the presence of hyphae.

For the other *S. aureus* genes, no difference between their expression on hyphae-poor and hyphae-rich models was observed. The expression of *atl* was globally increased. This autolysin is known to promote attachment to polystyrene surfaces and plays an important role in biofilm development (Houston et al. 2011). It also favours adhesion to *C. albicans* hyphae (Schlecht et al. 2015). Like the haemolysins, *psmα* is directly stimulated by the *agr* operon. However, its expression remained low, especially in the hyphae-rich model at 28 h, and increased in both models at longer incubation times. This gene encodes a surface-active peptide that can aid biofilm formation at low concentrations through the formation of amyloids together with extracellular DNA. It also greatly contributes to matrix disaggregation at higher concentrations, which is coherent with the increase in expression observed over time (Schwartz et al. 2016; Zheng et al. 2018). The up-regulation of both *icaA* and *psmα* at 48 h may indicate an equilibrium between matrix formation and dispersal in mature biofilms. Lastly, the two stress-induced regulators for *S. aureus*, *sigB* and *rsh*, showed almost no difference compared with their expression in planktonic cells, which indicates that *S. aureus* cells were not under stressful conditions in the biofilms.

E. coli, *fimA* and *pgaC*, involved in adhesion and PNAG synthesis, respectively, were consistently up-regulated in both early-stages and mature biofilms. Blumer et al. (2005) showed that type-1 fimbriae are essential for adhesion to abiotic surfaces. As opposed to what was observed for *S. aureus,* the *E. coli* stress-induced regulators *rpoS* and *relA* were overexpressed, indicating that *E. coli* cells are in highly stressful conditions, both in hyphae-rich and hyphae-poor biofilms. However, the expression of *relA* was generally higher in hyphae-poor biofilms, suggesting that *E. coli* was slightly less stressed in the presence of hyphae, although Peters et al. (2010) reported that *S. aureus* is more likely to adhere to hyphae and form biofilm than *E. coli*. Also, the stressful conditions created by the environment of the present mixed-species biofilms for *E. coli* might be the cause of the high variability in the number of culturable cells observed among experiments.

The present model suffers from some limitations. First, it is an *in vitro* model cultured in optimal conditions, i.e. with a rich medium, in aerobic conditions, and without an immune system. Second, the polystyrene surface of 96-wells plates is not clinically relevant, although it allowed optimisation of the culture conditions. Ti coupons provide a more relevant surface, but the other limitations remain the same. Third, the model does not allow the study of early-stage biofilms, as the growth of *C. albicans* was forced before the bacteria in the hyphae-rich protocol and possible population dynamics are not considered. Fourth, it examines reference strains only, which may not represent the diversity in biofilm formation capacities observed in clinical isolates, but this was the necessary step for developing a new model.

In summary, this article proposes an endpoint model of a polymicrobial mature biofilm infection that emphasises the co-localization and gene expression of cross-kingdom microorganisms. The culture conditions were shown to have a major impact on hyphal morphogenesis and therefore on the resulting biofilm. Biofilms enriched in hyphae had larger biomass, probably related to the overexpression of the enzymes responsible for the synthesis of exopolysaccharides. The co-cultured bacteria also synthesised more virulence-related molecules, which might contribute to explaining the higher pathogenicity of polymicrobial biofilms.

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Disclosure statement

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In vitro polymicrobial inter-kingdom three-species biofilm: influence of hyphae on biofilm formation and bacterial physiology

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Supplemental information

Supplemental Results

Growth conditions optimization

The growth conditions were optimized for the three-species biofilm with reference strains of *S. aureus*, *E. coli* and *C. albicans*. The aim was to set up a protocol that allowed culturing a biofilm with high biomass and containing more bacteria than fungi, with no overgrowth of one of the bacterial species over the other, and remaining stable for at least 72 h. The development of the model was performed first with all the species added at the same time.

Preliminary results showed that culturing the biofilm in a non-buffered medium led to an acidification of the medium below pH 5.5, which was detrimental to bacterial growth, notably *S. aureus* (data not shown). Thus, maintaining the pH of the culture medium neutral to slightly acidic was crucial to the model.

Several buffer systems were tested in RPMI + 1 % glucose and the results are represented in Figure S1. Biofilms cultured with either phosphate or Hepes buffers showed the more abundant biomass at 48 h. Yet only the phosphate buffer at the largest concentration tested (50 mM $H_2PO_4^-$ / 74.1 mM HPO_4^{2-}) was able to maintain the pH above 6 from 24 to 72 h and was thus selected for further experiments.

Other conditions tested to increase the nutrients available for the biofilm and to boost its growth were the removal of non-adherent cells and the supplementation of the media with an iron source or FBS. Results are shown in Figure S2. The two first modifications showed no significant improvement and they were discarded. The latter allowed an increase in biomass at 72 h only. Nevertheless, the supplementation of FBS was avoided in a first attempt because this model could be used for testing the activity of antimicrobial compounds, some of which could bind to serum proteins.

Inoculum adjustment

Several combinations of the relative initial inocula were tested for the three species. Ratios of inoculum are expressed in multiples of 2.5 $x10^6$ cfu ml^{-1} to facilitate the comparison between conditions. Biomass reached similar levels at 48 h (Figure S3), with absorbance ranging between 0.75 to 1.25 AU cm^{-2}. The lowest biomass levels were observed for the highest *E. coli* inoculum tested (6.53 $x10^7$ cfu ml^{-1}; 12:26:2 *S.aureus:E.coli:C.albicans*), and, to some extent, for the lowest inoculum of *C. albicans* (1.1 $x10^6$ cfu ml^{-1}; 0.1:0.6:0.4 *S.aureus:E.coli:C.albicans*). In this case, absorbance values were also very variable at 48 h.

Conversely, changing the inoculum and the relative proportions of the three species allowed us to modulate the resulting culturable cells in the biofilms (Figure S4). At inocula of *C. albicans* $\geq$ 5 $x10^6$ cfu ml^{-1} (Figure S4 A-F) and of *S. aureus* 2-4 fold higher than those of *E. coli*, *S. aureus* overgrew *E. coli*, resulting also in an abundance of *E. coli* cells lower than that of *C. albicans* at 48 h (Figure S4 A-D). To the opposite, with an inoculum of *E. coli* 2-3 times higher than that of *S. aureus* (Figure S4 E-F), *E. coli* abundance was higher than that of *C. albicans* only when *E. coli* inoculum was drastically increased up to 6.53 $x10^7$ cfu ml^{-1} (relative ratios of 12:26:2; compare Figure S4 E *vs*. F). Nevertheless, the abundance of *C. albicans* and the biomass (Figure S3) were greatly reduced at 48 h in these conditions, which were therefore not retained as a suitable option. At inocula of *C. albicans* below 5 $x10^6$ cfu ml^{-1} (Figure S4 G-L) and of *S. aureus* 40 times lower than those of *E. coli* (Figure S2 G), the abundance of *S. aureus* cells was lower than that of *C. albicans* at 48 h. For the other bacterial inocula (Figure S4 H-L), the abundance of both bacteria was similar or higher than that of *C. albicans*.

Finally, the inoculum of 15:6:2.5 $x10^6$ (*S.aureus:E.coli:C.albicans*; Figure S4 J and Figure 4 A) was selected as it showed the most stable culturable cells values up to 48 h.

Figure S1. Biomass and pH for 3-species biofilms of *S. aureus*, *E. coli* and *C. albicans* cultured in RPMI + 1 % glucose and different buffer systems (concentrations expressed in mM). The inoculum in all conditions was of 81:54:3 x10^5 cfu ml^{-1}. Cit: citric acid. TM: Tris/maleate. Dotted line: target lower limit of pH. Red box: buffer selected for further experiments. n = 3 (excepted or 20/30 mM and 50/74 mM $H_2PO_4^-/HPO_4^{2-}$: n = 6).

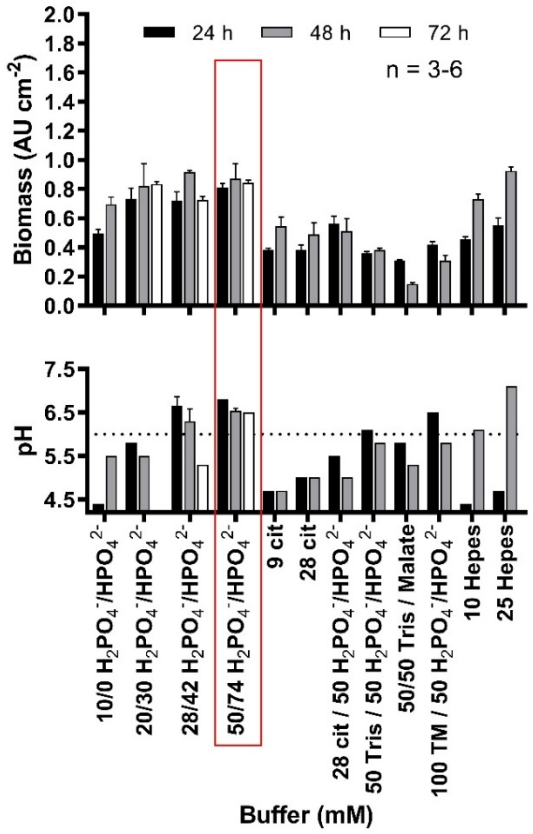

Figure S2. Biomass for 3-species biofilms of *S. aureus*, *E. coli* and *C. albicans* cultured in RPMI + 1 % glucose + 50/74 mM $H_2PO_4^-/HPO_4^{2-}$ (RGP) with different culture conditions. The inoculum was of 81:54:3 x10^5 cfu ml^{-1} in A and C, and 150:60:20 x10^5 cfu ml-1 in B. A: removal of non-adherent cells. B: supplementation of an iron source (mM). AmFeS: ammonium iron (II) sulphate, $(NH_4)_2Fe(SO_4)_2$. FePP: ferric (III) pyrophosphate, $Fe_4(P_2O_7)_3$. KCyFe: potassium hexacyanoferrate (II), $C_6N_6FeK_3$. C: supplementation of foetal bovine serum (FBS). Different lowercase letters (a/b), symbols (°/*/^) or capital letters (A/B) highlight significant differences among conditions at 24, 48 or 72 h, respectively, non-parametric One-way ANOVA + Dunns test, p-value <0.05.

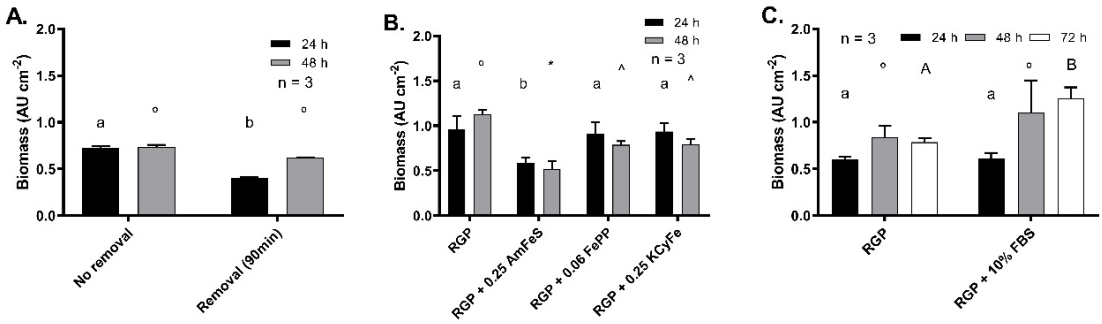

Figure S3. Biomass for 3-species biofilms of *S. aureus*, *E. coli* and *C. albicans* cultured in RPMI + 1 % glucose + 50/74 mM $H_2PO_4^-/HPO_4^{2-}$ (RGP) with different inocula. Inocula are written as estimated ratios of *S.aureus:E.coli:C.albicans* in multiples of 2.5 x10^6 cfu ml^{-1}. Red box: inoculum of 15:6:2.5 x10^6 cfu ml-1 selected for further experiments.

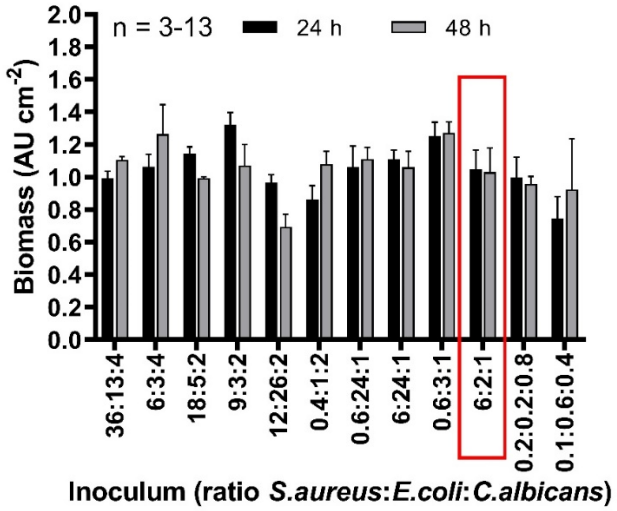

Figure S4. Culturable cells of tested 3-species biofilms for the optimization of the inoculum of *S. aureus* (green), *E. coli* (red) and *C. albicans* (blue), cultured in RPMI + 1 % glucose + 50/74 mM $H_2PO_4^-/HPO_4^{2-}$ (RGP). Inocula are written as estimated ratios of *S.aureus:E.coli:C.albicans* with multiples of 2.5 x10^6 cfu ml^{-1}. Selected inoculum (J) is represented for clarity.

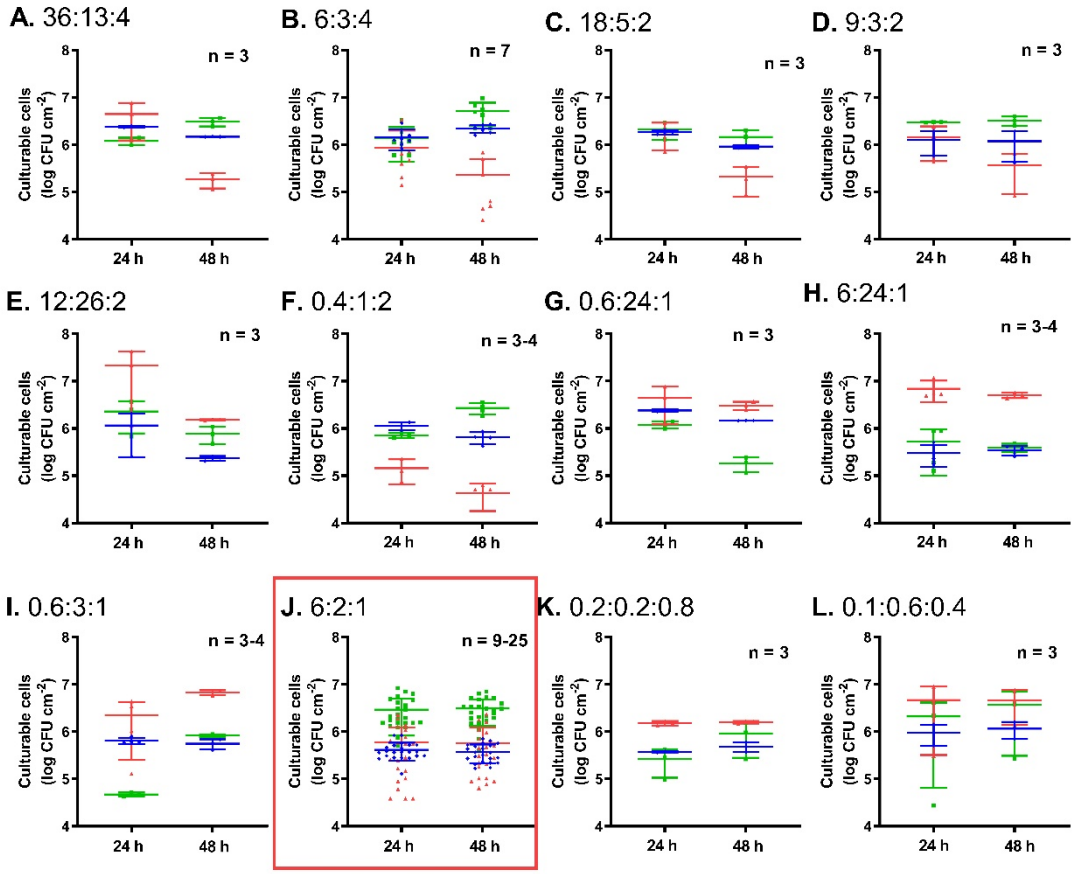

Figure S5. Expression of genes of interest in the dual-species *S. aureus : C. albicans biofilms* cultured with the hyphae-poor and hyphae-rich protocols. Sa: *S. aureus*. Ca: *C. albicans*. Housekeeping gene: *gmk*. Results are expressed as fold change relative to a pure planktonic culture in exponential growth. n=3. ns: no significant difference compared to the pure planktonic culture. One-way ANOVA, Tukey post-test.

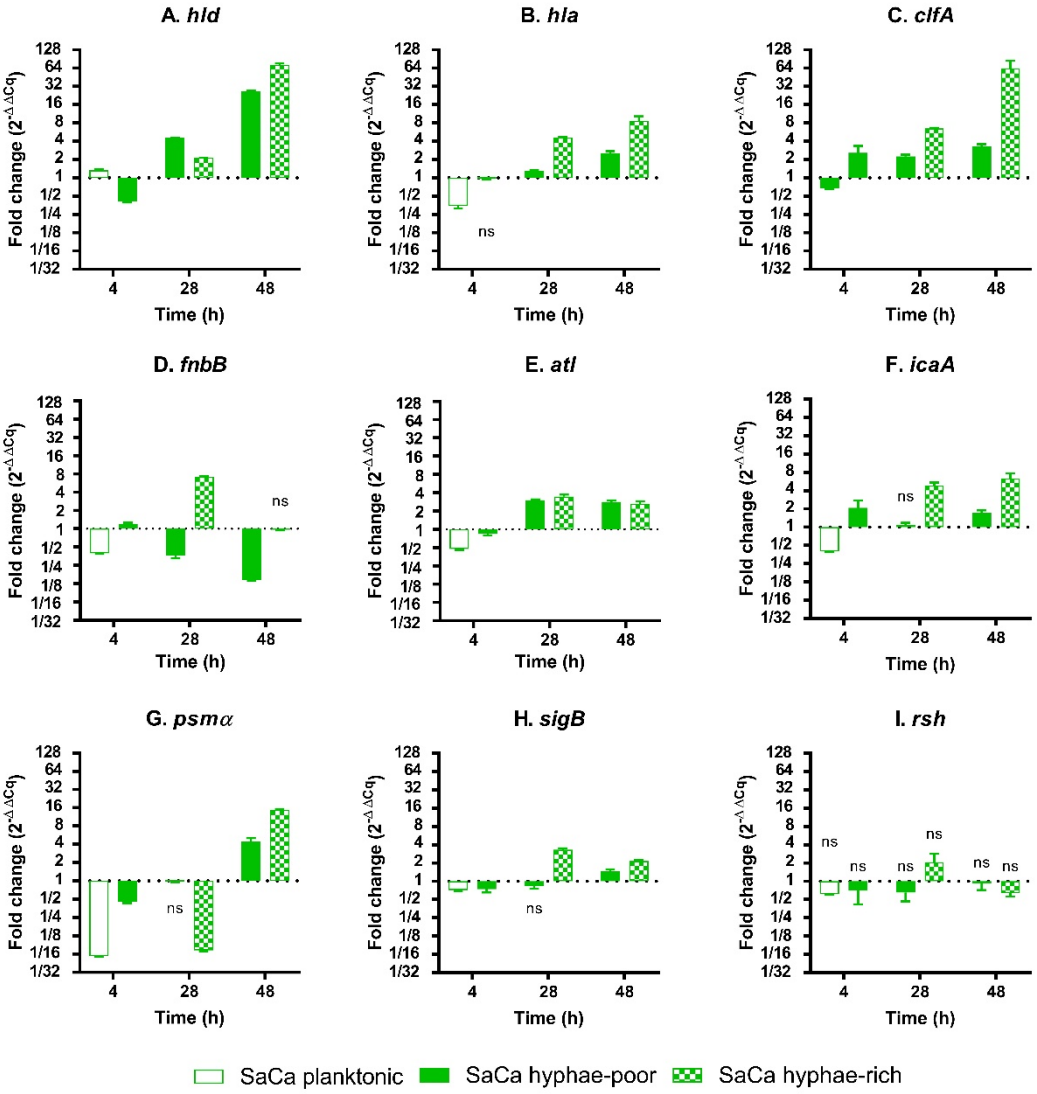

Figure S6. Expression of genes of interest in the dual-species *E. coli : C. albicans biofilms* cultured with the hyphae-poor and hyphae-rich protocols. Ec: *E. coli*. Ca: *C. albicans*. Housekeeping gene: *gapA*. Results are expressed as fold change relative to a pure planktonic culture in exponential growth. n=3. ns: no significant difference compared to the pure planktonic culture. One-way ANOVA, Tukey post-test.

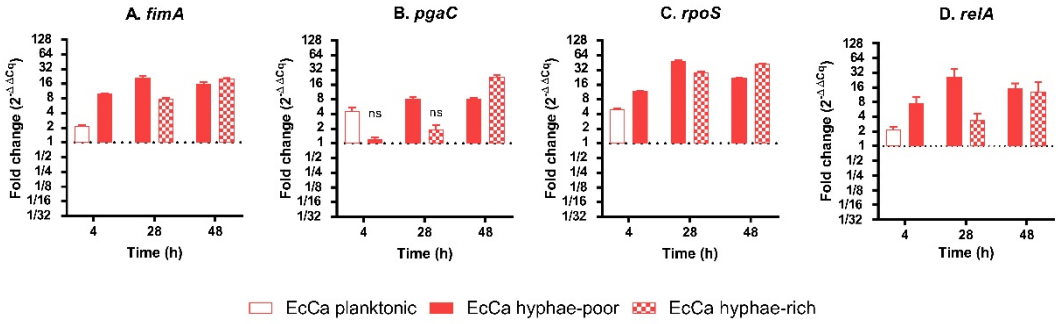

Table S1. Oligonucleotides used in this study. Sequences from Kempf, Trebesius and Autenrieth, 2000.

Name	**Microorganism(s)**	**Sequences 5'-3'** (Kempf et al. 2000)	**5' modification**	**Fluorescence (EX/EM, nm)**
Sta697-FITC	Staphylococci	TCC TCC ATA TCT CTG CGC	FITC	Green (490/525)
Ent168-Atto550	Enterobacteriaceae	CCC CCT CTT TGG TCT TGC	Atto550	Red (554/576)

Table S2. Primers used in this study.

Target	Gene	Forward	Reverse	Product (bp)	Reference
S. aureus	*gmk*	TCAGGACCATCTGGAGTAGGTAAA	TTCACGCATTTGACGTGTTG	108	Truong-Bolduc *et al.*, 2012
S. aureus	*clfA*	ACGAGTGACACAGGATCAGA	GTGAATTAGGCGGCACTACA	100	This article
S. aureus	*icaA*	CGAGAAAAAGAATATGGCTG	ACCATGTTGCGTAACCACCT	134	Siala *et al.*, 2016
S. aureus	*hla*	TGGGGACCATATGACAGAGAT	TCAAGGAAGTTCTCTGCTGC	101	This article
S. aureus	*hld*	GGAAGGAGTGATTTCAATGGCA	AGTGAATTTGTTCACTGTGTCG	88	This article
S. aureus	*psmα*	GCAGCAATTAATAATGACGGCG	ATTTACCTAGTAAACCTACGCCA	82	This article
S. aureus	*fnbB*	AGATACAAACCCAGGTGGCG	GTTGTATGGTCGCTCACTGC	108	This article
S. aureus	*atl*	TTCTATGGAGTGGGAGCTCTTG	TGAATGAAATCAGCACCGCC	116	This article
S. aureus	*sigB*	CTGATCGCGAACGAGAAATCA	GCCGTTCTTTGAAGTCTGGA	115	This article
S. aureus	*rsh*	CCCCAGCGAGTGATGTTATT	AATTTTGCCATTCACCTTGG	116	Peyrusson *et al.*, 2020
E. coli	*gapA*	GCCAGAACTGAATGGCAAAC	GCAGCTTTTTCCAGACGAAC	96	This article
E. coli	*fimA*	TAATGGTGGGACCGTTCACT	GCGGTACGAACCTGTCCTAA	105	Tapiainen *et al.*, 2012
E. coli	*pgaC*	AAATTCCCCATTTGCGGGTC	GCCATCAATGCACACCAGAT	110	This article
E. coli	*rpoS*	TTCGTTTGCCGATTCACATC	TCTCTTCCGCACTTGGTTCA	99	García-Heredia *et al.*, 2016
E. coli	*relA*	ATAAGCCGAGTGCCGAAGAG	GCCAACACCTTCGACTACCA	116	This article

Table S3. Specificity of the primers used for the target genes. Values are expressed as Cq (threshold=1000).

Target gene	**Planktonic 4h**	
	S. aureus	***E. coli***
gmk	20.74	39.46
hla	25.23	>40
clfA	25.91	>40
hld	20.27	39.43
icaA	28.98	>40
psmα	21.56	39.78
fnbB	20.14	31.07
atl	21.06	37.11
sigB	21.89	>40
rsh	20.72	36.31
gapA	>40	21.46
fimA	>40	25.09
pgaC	>40	30.54
rpoS	>40	24.78
relA	39.17	27.55

Table S4. Multiple comparisons for each gene tested among the different biofilm and planktonic samples. p-values and significance (Yes/No). One-way ANOVA, Tukey post-test. Sa: *S. aureus*. Ec: *E. coli*. Ca: *C. albicans*. SaEcCa, SaCa and EcCa: consequent co-inocula in planktonic (plank) or biofilm (HP or HP). HP: hyphae-poor biofilm. HR: hyphae-rich biofilm. n = 3.

	hld		***hla***		***clfA***		***fnbB***		***atl***	
Sa plank 4h vs. SaEcCa plank 4h	<0,0001	Yes	<0,0001	Yes	0.0255	Yes	*>0,9999*	*No*	<0,0001	Yes
Sa plank 4h vs. SaCa plank 4h	<0,0001	Yes	<0,0001	Yes	0.0397	Yes	<0,0001	Yes	<0,0001	Yes
Sa plank 4h vs. SaEcCa HP 4h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	0.0292	Yes
Sa plank 4h vs. SaEcCa HP 28h	*0.6806*	*No*	<0,0001	Yes	0.0025	Yes	<0,0001	Yes	*0.9505*	*No*
Sa plank 4h vs. SaEcCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Sa plank 4h vs. SaEcCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Sa plank 4h vs. SaEcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	0.0007	Yes	<0,0001	Yes
Sa plank 4h vs. SaCa HP 4h	<0,0001	Yes	*0.9096*	*No*	<0,0001	Yes	0.0003	Yes	0.0098	Yes
Sa plank 4h vs. SaCa HP 28h	<0,0001	Yes	0.0031	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Sa plank 4h vs. SaCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Sa plank 4h vs. SaCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Sa plank 4h vs. SaCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.9989*	*No*	<0,0001	Yes
SaEcCa plank 4h vs. SaCa plank 4h	<0,0001	Yes	*0.1112*	*No*	*0.9732*	*No*	<0,0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. SaEcCa HP 4h	<0,0001	Yes	0.0316	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. SaEcCa HP 28h	<0,0001	Yes	<0,0001	Yes	*0.8821*	*No*	<0,0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. SaEcCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. SaEcCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. SaEcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	0.0002	Yes	<0,0001	Yes
SaEcCa plank 4h vs. SaCa HP 4h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. SaCa HP 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. SaCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. SaCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. SaCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	>0,9999	No	<0,0001	Yes
SaCa plank 4h vs. SaEcCa HP 4h	<0,0001	Yes	*0.7444*	*No*	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaCa plank 4h vs. SaEcCa HP 28h	<0,0001	Yes	<0,0001	Yes	*0.8286*	*No*	<0,0001	Yes	<0,0001	Yes
SaCa plank 4h vs. SaEcCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*>0,9999*	*No*	<0,0001	Yes
SaCa plank 4h vs. SaEcCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaCa plank 4h vs. SaEcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaCa plank 4h vs. SaCa HP 4h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaCa plank 4h vs. SaCa HP 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.2755*	*No*	<0,0001	Yes
SaCa plank 4h vs. SaCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaCa plank 4h vs. SaCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaCa plank 4h vs. SaCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa HP 4h vs. SaEcCa HP 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.4717*	*No*
SaEcCa HP 4h vs. SaEcCa HP 48h	<0,0001	Yes	<0,0001	Yes	0.0003	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa HP 4h vs. SaCa HP 4h	<0,0001	Yes	<0,0001	Yes	*0.9732*	*No*	<0,0001	Yes	*>0,9999*	*No*
SaEcCa HP 28h vs. SaEcCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa HP 28h vs. SaEcCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa HP 28h vs. SaCa HP 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa HP 28h vs. SaCa HR 28h	<0,0001	Yes	*0.4276*	*No*	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes

	hld		*hla*		*clfA*		*fnbB*		*atl*	
SaEcCa HP 48h vs. SaEcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa HP 48h vs. SaCa HP 48h	<0,0001	Yes	*0.6465*	*No*	*0.0551*	*No*	<0,0001	Yes	*0.1008*	*No*
SaEcCa HR 28h vs. SaEcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa HR 28h vs. SaCa HR 28h	*0.2977*	*No*	<0,0001	Yes	*0.6828*	*No*	<0,0001	Yes	0.0004	Yes
SaEcCa HR 48h vs. SaCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaCa HP 4h vs. SaCa HP 28h	<0,0001	Yes	0.0026	Yes	*0.8821*	*No*	<0,0001	Yes	<0,0001	Yes
SaCa HP 4h vs. SaCa HP 48h	<0,0001	Yes	<0,0001	Yes	*0.2359*	*No*	<0,0001	Yes	<0,0001	Yes
SaCa HP 4h vs. SaCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaCa HP 28h vs. SaCa HP 48h	<0,0001	Yes	<0,0001	Yes	*0.0551*	*No*	<0,0001	Yes	*0.9558*	*No*
SaCa HP 28h vs. SaCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.0775*	*No*
SaCa HP 48h vs. SaCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.7089*	*No*
SaCa HR 28h vs. SaCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes

	icaA		*psma*		*sigB*		*rsh*	
Sa plank 4h vs. SaEcCa plank 4h	<0,0001	Yes	<0,0001	Yes	*0.9969*	*No*	*0.6232*	*No*
Sa plank 4h vs. SaCa plank 4h	<0,0001	Yes	<0,0001	Yes	0.0037	Yes	*0.4486*	*No*
Sa plank 4h vs. SaEcCa HP 4h	*0.8891*	*No*	<0,0001	Yes	<0,0001	Yes	*0.6109*	*No*
Sa plank 4h vs. SaEcCa HP 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*>0,9999*	*No*
Sa plank 4h vs. SaEcCa HP 48h	<0,0001	Yes	<0,0001	Yes	*>0,9999*	*No*	*>0,9999*	*No*
Sa plank 4h vs. SaEcCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.9892*	*No*
Sa plank 4h vs. SaEcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.1269*	*No*
Sa plank 4h vs. SaCa HP 4h	0.0004	Yes	<0,0001	Yes	0.0117	Yes	*0.7*	*No*
Sa plank 4h vs. SaCa HP 28h	*>0,9999*	*No*	*>0,9999*	*No*	*0.4024*	*No*	*0.5703*	*No*
Sa plank 4h vs. SaCa HP 48h	0.0087	Yes	<0,0001	Yes	0.0008	Yes	*>0,9999*	*No*
Sa plank 4h vs. SaCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.0842*	*No*
Sa plank 4h vs. SaCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.5529*	*No*
SaEcCa plank 4h vs. SaCa plank 4h	*0.5363*	*No*	<0,0001	Yes	0.0438	Yes	*>0,9999*	*No*
SaEcCa plank 4h vs. SaEcCa HP 4h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*>0,9999*	*No*
SaEcCa plank 4h vs. SaEcCa HP 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.5287*	*No*
SaEcCa plank 4h vs. SaEcCa HP 48h	<0,0001	Yes	<0,0001	Yes	*0.9585*	*No*	*0.684*	*No*
SaEcCa plank 4h vs. SaEcCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.0893*	*No*
SaEcCa plank 4h vs. SaEcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	0.0008	Yes
SaEcCa plank 4h vs. SaCa HP 4h	<0,0001	Yes	<0,0001	Yes	*0.1192*	*No*	*>0,9999*	*No*
SaEcCa plank 4h vs. SaCa HP 28h	<0,0001	Yes	<0,0001	Yes	*0.9475*	*No*	*>0,9999*	*No*
SaEcCa plank 4h vs. SaCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.877*	*No*
SaEcCa plank 4h vs. SaCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	0.0005	Yes
SaEcCa plank 4h vs. SaCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*>0,9999*	*No*
SaCa plank 4h vs. SaEcCa HP 4h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*>0,9999*	*No*
SaCa plank 4h vs. SaEcCa HP 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.3636*	*No*
SaCa plank 4h vs. SaEcCa HP 48h	<0,0001	Yes	<0,0001	Yes	0.0014	Yes	*0.5081*	*No*
SaCa plank 4h vs. SaEcCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	0.0491	Yes
SaCa plank 4h vs. SaEcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	0.0004	Yes
SaCa plank 4h vs. SaCa HP 4h	<0,0001	Yes	<0,0001	Yes	*>0,9999*	*No*	*>0,9999*	*No*
SaCa plank 4h vs. SaCa HP 28h	<0,0001	Yes	<0,0001	Yes	*0.5929*	*No*	*>0,9999*	*No*
SaCa plank 4h vs. SaCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.7361*	*No*

	icaA		*psma*		*sigB*		*rsh*	
SaCa plank 4h vs. SaCa HR 28h	<0,0001	Yes	0.0078	Yes	<0,0001	Yes	0.0002	Yes
SaCa plank 4h vs. SaCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*>0,9999*	*No*
SaEcCa HP 4h vs. SaEcCa HP 28h	0.0042	Yes	<0,0001	Yes	<0,0001	Yes	*0.5165*	*No*
SaEcCa HP 4h vs. SaEcCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.672*	*No*
SaEcCa HP 4h vs. SaCa HP 4h	0.024	Yes	<0,0001	Yes	<0,0001	Yes	*>0,9999*	*No*
SaEcCa HP 28h vs. SaEcCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*>0,9999*	*No*
SaEcCa HP 28h vs. SaEcCa HR 28h	0.0378	Yes	<0,0001	Yes	<0,0001	Yes	*0.9965*	*No*
SaEcCa HP 28h vs. SaCa HP 28h	0.0003	Yes	<0,0001	Yes	<0,0001	Yes	*0.477*	*No*
SaEcCa HP 28h vs. SaCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.1142*	*No*
SaEcCa HP 48h vs. SaEcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.1045*	*No*
SaEcCa HP 48h vs. SaCa HP 48h	<0,0001	Yes	<0,0001	Yes	0.0023	Yes	*>0,9999*	*No*
SaEcCa HR 28h vs. SaEcCa HR 48h	<0,0001	Yes	<0,0001	Yes	*0.5858*	*No*	*0.7302*	*No*
SaEcCa HR 28h vs. SaCa HR 28h	*0.2558*	*No*	<0,0001	Yes	<0,0001	Yes	*0.6053*	*No*
SaEcCa HR 48h vs. SaCa HR 48h	0.0006	Yes	<0,0001	Yes	*0.838*	*No*	0.0006	Yes
SaCa HP 4h vs. SaCa HP 28h	0.0019	Yes	<0,0001	Yes	*0.8609*	*No*	*>0,9999*	*No*
SaCa HP 4h vs. SaCa HP 48h	*0.9856*	*No*	<0,0001	Yes	<0,0001	Yes	*0.9213*	*No*
SaCa HP 4h vs. SaCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	0.0006	Yes
SaCa HP 28h vs. SaCa HP 48h	0.0375	Yes	<0,0001	Yes	<0,0001	Yes	*0.8404*	*No*
SaCa HP 28h vs. SaCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	0.0004	Yes
SaCa HP 48h vs. SaCa HR 48h	<0,0001	Yes	<0,0001	Yes	0.0004	Yes	*0.8272*	*No*
SaCa HR 28h vs. SaCa HR 48h	*0.6627*	*No*	<0,0001	Yes	0.0001	Yes	0.0004	Yes

	fimA		*pgaC*		*rpoS*		*relA*	
Ec plank 4h vs. SaEcCa plank 4h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	0.0053	Yes
Ec plank 4h vs. EcCa plank 4h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	*0.0577*	*No*
Ec plank 4h vs. SaEcCa HP 4h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Ec plank 4h vs. SaEcCa HP 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Ec plank 4h vs. SaEcCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Ec plank 4h vs. SaEcCa HR 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Ec plank 4h vs. SaEcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Ec plank 4h vs. EcCa HP 4h	<0,0001	Yes	*0.9988*	*No*	<0,0001	Yes	<0,0001	Yes
Ec plank 4h vs. EcCa HP 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Ec plank 4h vs. EcCa HP 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
Ec plank 4h vs. EcCa HR 28h	<0,0001	Yes	*0.0978*	*No*	<0,0001	Yes	0.0012	Yes
Ec plank 4h vs. EcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. EcCa plank 4h	*0.9879*	*No*	*>0,9999*	*No*	0.008	Yes	*0.9974*	*No*
SaEcCa plank 4h vs. SaEcCa HP 4h	<0,0001	Yes	*0.6047*	*No*	0.0005	Yes	0.0051	Yes
SaEcCa plank 4h vs. SaEcCa HP 28h	<0,0001	Yes	*0.998*	*No*	<0,0001	Yes	0.0001	Yes
SaEcCa plank 4h vs. SaEcCa HP 48h	<0,0001	Yes	*>0,9999*	*No*	0.0002	Yes	<0,0001	Yes
SaEcCa plank 4h vs. SaEcCa HR 28h	<0,0001	Yes	*>0,9999*	*No*	<0,0001	Yes	*0.1605*	*No*
SaEcCa plank 4h vs. SaEcCa HR 48h	<0,0001	Yes	*0.8621*	*No*	<0,0001	Yes	0.0032	Yes
SaEcCa plank 4h vs. EcCa HP 4h	<0,0001	Yes	0.0001	Yes	*0.888*	*No*	0.0099	Yes
SaEcCa plank 4h vs. EcCa HP 28h	<0,0001	Yes	*0.0524*	*No*	<0,0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. EcCa HP 48h	<0,0001	Yes	*0.0581*	*No*	0.0001	Yes	<0,0001	Yes
SaEcCa plank 4h vs. EcCa HR 28h	<0,0001	Yes	0.0499	Yes	<0,0001	Yes	*>0,9999*	*No*

	fimA		*pgaC*		*rpoS*		*relA*	
SaEcCa plank 4h vs. EcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
EcCa plank 4h vs. SaEcCa HP 4h	<0,0001	Yes	*0.8984*	*No*	<0,0001	Yes	0.0004	Yes
EcCa plank 4h vs. SaEcCa HP 28h	<0,0001	Yes	*>0,9999*	*No*	<0,0001	Yes	<0,0001	Yes
EcCa plank 4h vs. SaEcCa HP 48h	<0,0001	Yes	*>0,9999*	*No*	<0,0001	Yes	<0,0001	Yes
EcCa plank 4h vs. SaEcCa HR 28h	<0,0001	Yes	*0.9988*	*No*	<0,0001	Yes	0.0177	Yes
EcCa plank 4h vs. SaEcCa HR 48h	<0,0001	Yes	*0.9899*	*No*	<0,0001	Yes	0.0002	Yes
EcCa plank 4h vs. EcCa HP 4h	<0,0001	Yes	<0,0001	Yes	0.0001	Yes	0.0008	Yes
EcCa plank 4h vs. EcCa HP 28h	<0,0001	Yes	*0.1612*	*No*	<0,0001	Yes	<0,0001	Yes
EcCa plank 4h vs. EcCa HP 48h	<0,0001	Yes	*0.1761*	*No*	<0,0001	Yes	<0,0001	Yes
EcCa plank 4h vs. EcCa HR 28h	<0,0001	Yes	0.0142	Yes	<0,0001	Yes	*0.9112*	*No*
EcCa plank 4h vs. EcCa HR 48h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes
SaEcCa HP 4h vs. SaEcCa HP 28h	0.024	Yes	*0.9888*	*No*	*0.1851*	*No*	*0.932*	*No*
SaEcCa HP 4h vs. SaEcCa HP 48h	*0.5189*	*No*	*0.8267*	*No*	*>0,9999*	*No*	<0,0001	Yes
SaEcCa HP 4h vs. EcCa HP 4h	*0.2385*	*No*	<0,0001	Yes	0.0327	Yes	*>0,9999*	*No*
SaEcCa HP 28h vs. SaEcCa HP 48h	*0.9038*	*No*	*>0,9999*	*No*	*0.3696*	*No*	<0,0001	Yes
SaEcCa HP 28h vs. SaEcCa HR 28h	*0.9623*	*No*	*0.9689*	*No*	*0.621*	*No*	*0.1596*	*No*
SaEcCa HP 28h vs. EcCa HP 28h	<0,0001	Yes	*0.3441*	*No*	*0.0918*	*No*	*0.0516*	*No*
SaEcCa HP 28h vs. EcCa HR 28h	*0.998*	*No*	0.0048	Yes	*>0,9999*	*No*	0.0005	Yes
SaEcCa HP 48h vs. SaEcCa HR 48h	0.0018	Yes	*0.972*	*No*	<0,0001	Yes	<0,0001	Yes
SaEcCa HP 48h vs. EcCa HP 48h	0.0389	Yes	*0.128*	*No*	*>0,9999*	*No*	<0,0001	Yes
SaEcCa HR 28h vs. SaEcCa HR 48h	*0.7436*	*No*	*0.6465*	*No*	0.0002	Yes	*0.8701*	*No*
SaEcCa HR 28h vs. EcCa HR 28h	*>0,9999*	*No*	*0.1138*	*No*	*0.9359*	*No*	*0.4267*	*No*
SaEcCa HR 48h vs. EcCa HR 48h	<0,0001	Yes	<0,0001	Yes	*0.9623*	*No*	*0.9403*	*No*
EcCa HP 4h vs. EcCa HP 28h	<0,0001	Yes	<0,0001	Yes	<0,0001	Yes	0.0006	Yes
EcCa HP 4h vs. EcCa HP 48h	0.0114	Yes	<0,0001	Yes	0.0091	Yes	*0.1321*	*No*
EcCa HP 4h vs. EcCa HR 28h	*0.6645*	*No*	*0.4854*	*No*	<0,0001	Yes	0.0397	Yes
EcCa HP 28h vs. EcCa HP 48h	*0.2577*	*No*	*>0,9999*	*No*	0.0003	Yes	*0.5595*	*No*
EcCa HP 28h vs. EcCa HR 28h	<0,0001	Yes	<0,0001	Yes	0.0223	Yes	<0,0001	Yes
EcCa HP 48h vs. EcCa HR 48h	*0.5322*	*No*	0.0016	Yes	0.0038	Yes	*0.9892*	*No*
EcCa HR 28h vs. EcCa HR 48h	<0,0001	Yes	<0,0001	Yes	*0.2146*	*No*	0.0003	Yes

Table S5. Summary of literature data for multispecies biofilms for which data are provided regarding the culturable surface (bottom + walls). The last column shows the multipliers used to convert these results per unit of surface, based on references provided in the corresponding article.

Reference	Species	Media	Model[a]	Culture volume[c] (mL)	Culturable surface (cm^2)	Suspension volume[d] (mL)	Multiplier ($cfu\ ml^{-1}$ to $cfu\ cm^{-2}$)
Harriott and Noverr, 2010	*S. aureus : C. albicans*	BSA50%	96w F	0.11	1.01	0.1	0.1
Kart *et al.*, 2013	*S. aureus : P. aeruginosa : C. albicans*	BHI + BSA5%	96w U[b]	0.1	0.95	0.1	0.11
De Brucker *et al.*, 2015	*E. coli : C. albicans*	RPMI + MOPS	96w F	0.1	0.95	0.1	0.11
Zago *et al.*, 2015	*S. aureus : C. albicans*	RPMI	96w F	0.15	1.26	0.1	0.08
Kong *et al.*, 2016	*S. aureus : C. albicans*	RPMI + Hepes	96w F	0.2	1.57	0.1	0.06
This article	*S. aureus : E. coli : C. albicans*	RPMI + PO_4 + Glc RPMI + Hepes	96w F	0.2	1.57	0.2	-

[a]Diameter of the well: 0.64 cm

[b]Estimated as flat-bottomed for convenience.

[c]Volume of the medium during the incubation.

[d]Volume in which the biofilm has been resuspended.

References to the supplementary material

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