

Hyphal growth determines spatial organization and coexistence in a pathogenic polymicrobial community in a spatially structured environment

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Running title: Hyphal growth shapes polymicrobial structure

Abstract

The bodies of macroorganisms host microbes living in multi-species communities. Sequencing approaches have revealed that different organs host different microbiota and tend to be infected by different pathogens, drawing correlations between environmental parameters at the organ level and microbial composition. However, less is known about the microscale dimension of microbial ecology, particularly during infection. In this study, we focus on the role of microscale spatial structure, studying its influence on the ecology of a polymicrobial infection of *P. aeruginosa*, *S. aureus*, and *C. albicans*. Although these pathogens are commonly found together in the lungs of chronically ill patients, it is unclear whether they coexist or compete and segregate in different niches. We find that, whereas *P. aeruginosa* quickly outcompetes *C. albicans* and *S. aureus* on large surfaces, robust spatial organization and coexistence emerges in spatially structured microenvironments. In confined spaces, slowly growing *C. albicans* is able to leverage rapid radial hyphal growth to conquer

boundaries, where it establishes itself displacing the other pathogens. Similar outcomes are observed when the *P. aeruginosa* strain carries *mexT*-inactivating mutations, which are often found in clinical isolates. The observed spatial organization enables coexistence and potentially determines infection severity and outcomes. Our findings reveal a previously unrecognized role of mechanical forces in shaping infection dynamics, suggesting that microenvironmental structure might be a critical determinant of pathogen coexistence, virulence, and treatment outcomes. Because adaptations, such as changes in morphology, are widespread among microbes, these results are generalizable to other ecologies and environments.

Keywords: Microbial ecology; Infection; Soft matter; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; *Candida albicans*; Microenvironmental structure; Mechanical interactions; Microfluidics; Pathogen coexistence

1 Introduction

2 “Omic” studies have revealed that microbes seldom live in isolation, both in the
3 healthy [1–3] and diseased body [4–6]. Instead, many different species come to-
4 gether to consume substrates giving rise to polymicrobial communities and rich
5 ecologies [7–9]. Interactions with different structural characteristics of their en-
6 vironments produce spatial patterning that in “macroorganisms” we commonly
7 call biogeography [10]. This patterning on both the macro and microscale even-
8 tually determines the phenotypic outcome of the polymicrobial community, ei-
9 ther in terms of virulence [11–16], survival to treatment [17–20], or substrate
10 transformation [21–24].

11 Although we have an understanding of macroscale factors thanks to “omic”
12 approaches, microscale ones such as biotic factors and ecological interactions
13 require highly spatially and time resolved assays [25] which until recently have
14 been hard to perform with the same techniques [26]. These have been studied
15 mainly through mathematical modeling [27, 28] or in synthetic communities
16 grown in the lab [29–33]. In addition to revealing a wealth of new ecology, these
17 “synthetic” communities have the merit of enabling live direct observations.

18 Microscopy complements omic approaches, offering high spatial and time
19 resolution and an access to mechanistic understanding of microscale phenom-
20 ena. Despite progress in biomedical imaging, the observation of these microbial
21 consortia *in vivo* in humans remains a challenging task. Some indications on
22 the shapes and forms of these communities are provided by *ex vivo* observa-
23 tions. These images have been fundamental to understanding that microbes
24 can indeed have complex spatial organizations at the microscale - an “urban
25 biogeography” - that can have significant consequences on their pathogenicity
26 and biophysical and metabolic properties [22, 34]. Understanding how spe-
27 cific “urban biogeographies” come about and mapping different organizations
28 to their emergent properties will open up new intervention strategies aimed at
29 preventing and treating pathogenic scenarios, including infection. To achieve
30 this goal, approaches that encompass the host, polymicrobial communities, live

microscopy, and controlled environments are needed. Currently, two model scenarios satisfy these requirements in different measures and their choice depends on the research question: animal models and infection-on-chip. Animal models offer the most realistic conditions as they respond to infection with a complete immune system and therefore are well suited to study the complex interaction between microbes and host response [35–37]. However, imaging infection in these systems is challenging [38]. Even in transparent hosts, such as zebrafish larvae, the spatial resolution for live microscopy is limited to the organ level [39–45]. Organoids and infection-on-chip systems that encompass host tissues strike a compromise, improving imaging to the detriment of the immune response, which tends to be only partial [46–51]. In both cases, complexity remains high and isolating the mechanistic contribution of specific parameters can be challenging.

In this work, we focus on the role of spatial structure in the lung on shaping the microscale ecology of a respiratory pathogenic polymicrobial community. A large body of work [52–54] has demonstrated that microbial phenotype and evolution are conditioned by the mechanical properties of the substrate, both passively [55] and through active sensing [56, 57]. Contact with surfaces is commonly associated with the formation of mono- and polymicrobial biofilms [58, 59], which are studied almost exclusively on surfaces that do not impose limitations in the surface plane [60]. In these settings, spatial organization within biofilms has been studied as an emergent property of microbe-microbe interactions, with surfaces acting as a point of anchorage that limits growth and movement in the direction perpendicular to the surface [13, 61–64]. The microenvironments within host which microbes inhabit are often more complex than simple planar surfaces, and thus these models neglect important mechanical contributions [55, 65]. Because of these limitations, questions regarding the impact of the mechanical and spatial characteristics of the host microenvironment on the ecology of polymicrobial communities remain open. For example, it is unclear why the trio of microbes *P. aeruginosa*, *S. aureus*, and *C. albicans*, that are difficult to co-culture in the lab [66], are consistently found together in the sputum of patients, particularly those affected by chronic diseases such as cystic fibrosis [67–71]. Do different species in the lung coexist or are they segregated in different regions of the organ?

To investigate the impact of spatial structure on microbial ecology, we compare the spatial organization of polymicrobial communities of *P. aeruginosa*, *S. aureus*, and *C. albicans* in two scenarios: unconfined surfaces and geometrical alveoli mimics. With the latter, we expand the definition of an infection-on-chip, producing microfluidics that do not contain host cells but that reproduce the biochemical properties of the infection site and its key spatial features. This allows us to specifically control and isolate the impact of confinement on ecology, bypassing confounding factors that emerge from microbe-host interactions or from the requirements of host cell culturing. We find that, although *P. aeruginosa* quickly outcompetes the other species on an unlimited surface, alveoli-like confinement profoundly alters ecology, allowing persistent polymicrobial cohabitation. In the geometrical alveoli mimics, we observe the robust

77 emergence of a spatial organization that enables co-existence. Despite growing
78 slower than the other species, *C. albicans* is able to conquer the edges and the
79 closed end of the microcompartments. We demonstrate that this is enabled
80 by a transition to hyphal morphology that inherently focuses directional radial
81 growth. Upon reaching a barrier, *C. albicans* tends to bend and reorient, con-
82 tinuing growth along the edge and displacing other microbes, as reproduced in
83 our purely biomechanical agent-based simulations. Finally, we probe the ro-
84 bustness of the observed spatial organization using a clinically relevant *mexT*
85 mutant of *P. aeruginosa* in which certain virulence factors such as motility and
86 pigments production [72] are overexpressed. Taken together, our results reveal a
87 previously unrecognized role of mechanical forces in shaping infection dynamics
88 and suggest that the microenvironmental structure is a critical determinant of
89 pathogen coexistence, virulence, and treatment outcomes.

90 Materials and Methods

91 Microbial strains, media, and preculture conditions

92 For *P. aeruginosa*, we used two strains: a PA01 reference strain expressing eCFP
93 [73] from the chromosome and a spontaneous *mexT* frameshift mutant emerging
94 in the same background (and therefore also expressing eCFP). We characterize
95 the strains using whole genome sequencing (MicrobesNG, UK). For *S. aureus*
96 we use a SH1000 strain expressing eGFP from the chromosome [74]. Our ref-
97 erence strain for *C. albicans* is CAF2.1-dTom-NATr that expresses dTomato
98 under the control of the pENO1 enolase promoter [75]. The yeast-locked strain
99 is the WYZ12.2 *hgc1* Δ/Δ , a CAF2.1 derivative from [76]. Prior to any of the
100 experiments, single cultures of *P. aeruginosa*, *S. aureus*, and *C. albicans* are
101 grown overnight in lysogeny broth (0.5% yeast extract, 1% Bacto Tryptone,
102 0.05% NaCl) in a shaking (220 rpm) incubator at 37°C. 1 ml aliquots of each
103 culture were washed twice in PBS (0.8% NaCl, 0.02% KCl, 1.44% Na₂HPO₄,
104 0.24% KH₂PO₄, pH 7.4) with centrifugation (8000xG, 1 min). The single
105 colonies used to inoculate the overnights are streaked on LB plates from frozen
106 stocks on the day before inoculation for *P. aeruginosa* and up to a week before
107 for *S. aureus* and *C. albicans*. Except where otherwise indicated, all of our ex-
108 periments are carried out in ASM, of which we give a recipe in SI. Our ASM is
109 prepared as in [66] and is therefore a modified version of previously published
110 media [77–79].

111 Soft agarose experiments

112 4 mm thin agarose plates are produced by filling the lids of 3.5 cm petri dishes
113 (Greiner, UK) with 3 ml molten 1.5% agarose solutions in ASM. The plates
114 are seeded with 0.2 μ l mixtures of the washed *P. aeruginosa*, *S. aureus*, and
115 *C. albicans* at various dilution ratios. To prevent agarose marking with the tip
116 of the pipette during seeding, we slowly pipette out the small culture volume

117 until a drop is visible at the end of the tip. The edges of the plates are secured
 118 to a custom-made microscope mount with double-sided tape and immediately
 119 imaged. This is carried out using a Nikon 10 $\times$ Plan Apo air objective with a
 120 numeric aperture of 0.45 on a Nikon Ti-E microscope using Nikon Perfect Focus
 121 System. A total of 280 images per colony were collected using a Teledyne FLIR
 122 BFS-U3-70S7M-C with a 7.1 MP Sony IMX428 monochrome image sensor. All
 123 images are captured at 3208 $\times$ 2200 pixels, with a resolution of 0.43 μm /pixel.
 124 Each FOV is imaged 7 times, of which 1 in brightfield and 3 in 3 fluorescence
 125 channels, each imaged at two different exposure time and gain combinations:
 126 0.5 s, 20 gain and 0.2 s, 10 gain for dTomato; 0.2 s, 32 gain and 0.005 s, 32
 127 gain for eGFP; 0.06 s, 32 gain and 0.005 s and 32 gain for eCFP. Illumination
 128 is provided by color Light Emitting Diodes: red for brightfield (Lumileds Lux-
 129 eon Z LXZ1-PD01), indigo for eCFP (Lumileds Luxeon UV LHUV-0425), blue
 130 for eGFP (Lumileds Luxeon Z LXZ1-PB01), and lime for dTomato (Lumileds
 131 Luxeon Z LXZ1-PX01). For fluorescence imaging we use Semrock optical fil-
 132 ter sets (IDEX Health and Science, USA), CFP-2432C for eCFP, GFP-3035D
 133 for eGFP and TxRed-4040C for dTomato. After imaging, which typically took
 134 2 hours, positions of the colonies on the plates are recorded and the plates are
 135 temporarily sealed with a second lid glued with Covergrip coverslip sealant (Bi-
 136 otium, USA) to maintain moisture. The plates are transferred to a static 37°C
 137 incubator for 24 hours and imaged again.

138 Microfluidic lung mimic production

139 Microfluidic master molds are produced in-house using SU8 (Kayaku AM, USA)
 140 soft-lithography following the manufacturer’s guidelines. A first thin layer of
 141 SU8-2010 is deposited on a 10.16 cm (4 inches) silicon wafer (PI-KEM, UK),
 142 exposed to UV light in a MicroWriter ML3 Pro (Durham Magneto Optics, UK)
 143 and baked to obtain a final thickness of approximately 8 μm (Fig. 2C). A second
 144 layer of SU8-2075 is then deposited on the mold, exposed to UV light, baked
 145 and washed with SU8 developer (Kayaku AM, USA) to obtain the main trench
 146 (Fig. 2A), with a height of 0.3 mm. The designs are inspired by the family
 147 machine [80]. The molds are then used to fabricate microfluidic chips using
 148 degassed PDMS at a 1:10 ratio between curing agent and elastomer. Curing of
 149 the PDMS was performed in a 60°C oven. Before use, chips are released from
 150 the mold using a scalpel, inlets and outlets are punched using a biopsy puncher
 151 with 0.77 mm internal diameter (World Precision Instruments-Europe, UK),
 152 the chip is plasma bonded to a 0.145 mm thick glass coverslip (VWR, UK) and
 153 baked at 60°C for at least 10 minutes. For the microfabrication of a mother
 154 machine that could house *C. albicans*, we use the same procedure, but lower the
 155 height of the first SU8 layer to 6 μm .

156 Lung-mimic experiments preparation

157 Microfluidic channels are loaded with microbes mixtures obtained from the
 158 washed overnight cultures and inspected using microscopy. To carry out the

experiment, inlets and outlets of the microfluidic chips are connected to Tygon tubing (Cole Parmer; 0.020" $\times$ 0.060" outside diameter), using 90° bent connectors (Intertronics, UK; stainless steel, 0.89-0.58 OD-ID, 20 gauge) as before [81]. A 20 μ /min flow of ASM medium is prompted by a syringe pump (kdScientific, USA), loaded with a 60 ml plastic syringe (BD plastipak, UK), connected to the tubing via blunt needles (Intertronics, UK; stainless steel, straight blunt, 1/2", 23 gauge).

Lung-mimic experiments

Experiments are carried out at 37°C by housing the microfluidic chip in custom-built heating elements that are fitted to the automated xy stage of a Nikon Eclipse Ti-E. Before starting the experiment, and after loading, an arbitrarily fast flow pulse of fresh ASM medium is delivered to the main trench of the chip to remove microbes that had not entered in the alveoli-mimicking boxes. Imaging is automatically carried out in two phases. In the first phase, we imaged the 100 boxes on 1 side of the channel using a Nikon 40 $\times$ CFI Plan Fluor air objective with a numeric aperture of 0.75. Each box is imaged 19 times, once in brightfield and 18 times in fluorescence using the same LEDs and filters given in the *Soft agarose experiments* section. Each fluorescence channel is imaged at maximum gain at three different z offsets, with two exposure times: 0.4 s and 0.1 s for dTomato; 0.05 s and 0.003 s for eGFP; 0.5 s and 0.05 s for eCFP. In the second phase, we image the whole channel using the 10x air objective described in the *Soft agarose experiments* section. At this stage, we collect 266 images per channel for a total of 38 FOVs, each imaged 7 times: once in brightfield and twice per each fluorescence channel, using 32 gain and different exposure times: 0.5 s and 0.2 s for dTomato; 0.05 s and 0.005 s for eGFP; 0.06 s and 0.005 s for eCFP. The two phases combined typically take 54 minutes of microscope time and are automatically repeated every hour.

Mother machine experiments

To evaluate the correspondence between biomass and fluorescence along various growth stages in the three strains we use mother machines. This allows us to track fluorescence in single cells, while extracting their biomass based on their cross-sectional area. Because the cells are confined within pistons, the cross-sectional area provides a good approximation of the volume, as cells cannot stack on top of each other and are uniformly oriented. This allows us to characterize fluorescence with the same system used for data collection. In addition, it allows us to bypass the issues associated with bulk measurements of microbes that change morphology, that form clumps and biofilms, and that secrete fluorescent pigments (SI Fig. 4D). In particular, we use two mother machines: one with pistons with width and height below 2 μ m [81] for *P. aeruginosa* and *S. aureus* and one with pistons with width and height below 6 μ m for *C. albicans*. Before loading the cells in the pistons, the channels are incubated overnight at 37°C with a 2% solution of bovine serum albumin (BSA). Loading is carried out from

201 50x concentrated LB overnight cultures, double-washed in PBS in all cases.
 202 For *P. aeruginosa* and *S. aureus* loading occurred spontaneously, whereas for
 203 *C. albicans* we enhance it by spinning the mother machine at 1000 rpm in a
 204 spin coater for 1 minute. Once loading is satisfactory, the inlet and outlet of
 205 the mother machine are attached to a syringe pump, as described above, using
 206 a 5 ml syringe (BD plastipak, UK). The chip is heated to 37°C and ASM is
 207 flowed in at 5 μ l/min. Further explanations on the extraction of fluorescence
 208 and biomass are given in SI.

209 Data analysis

210 Details on data analysis and simulations are given in SI.

211 Results

212 *P. aeruginosa* quickly outcompetes *S. aureus* and *C. albi-* 213 *cans* on a surface

214 We consider a lung-relevant pathogenic polymicrobial community of *P. aerugi-*
 215 *nosa* (PA), *S. aureus* (SA), and *C. albicans* (CA) on the simplest and best stud-
 216 ied [23] example of a structured environment: a soft agarose surface. The species
 217 in our community, express different fluorescent proteins that allow their track-
 218 ing in epifluorescence microscopy: eCFP for PA, eGFP for SA, and dTomato
 219 for CA. The strains are PA01 for PA, SH1000 for SA, and CAF2.1 for CA. To
 220 recapitulate aspects of lung biochemistry, plates are composed of artificial spu-
 221 tum medium (ASM) [66]. Using a micropipette, we seed small aliquots of cells
 222 from the three species mixed in different ratios (1:1:1, 1:0.1:0.1, 1:0.01:0.01) and
 223 observe their growth at 37°C after 24 hours. When the inoculum contained the
 224 same number of cells of the three species, despite the much lower cell size and
 225 starting biomass, *P. aeruginosa* dominated the mixed colony after 24 hours. The
 226 colonies nearly doubled their radii over the time interval. Whereas *C. albicans*
 227 and *S. aureus* show limited growth and remain confined to their seeding spots,
 228 all of the radius gained by the colony over the 24 hours is due to *P. aeruginosa*
 229 growth (Fig. 1A). This is consistent with reports indicating that motility is a
 230 major driver of growth on surfaces [82–85]. To understand the extent of such
 231 advantage, we repeat the experiment at two more PA:SA:CA seeding ratios:
 232 0.1:0.1:1 and 0.01:0.01:1. Reducing the PA titer by 10 times does not signifi-
 233 cantly affect the colony growth phenotype observed, with PA still dominating
 234 (Fig. 1B). The phenotype switches to CA dominance when its initial titer is 100
 235 times higher (Fig. 1). The colony radius also doubles over 24 hours, confirming
 236 competitive inhibition between PA, CA, and SA, as previously observed [66].

Spatial organization emerges within 10 hours in a lung mimic

Agar surfaces, such as the one we used, model situations in which space is unlimited in the planar surface and are therefore best suited to study unstructured environments such as the upper airways. The environment of the lower airways is significantly more structured, with alveolar volumes of the order of millions of cubic micrometers, which, if approximated to spheres, would measure 100 to 250 μm in diameter [86, 87]. To model the effects that spatial limits have on the ecology of our pathogenic polymicrobial community, we present a microfluidic model inspired by the geometrical constraints of the distal lung. Our design is similar to the family machine [80], and encompasses a central channel 1.5 cm long, 1 mm wide, 0.3 mm tall, lined on each side by 100 quasi-2D square geometries with a side length of 150 μm and a thickness of 8 μm (Fig. 2A and 2B). All measures are modeled on those of the distal lung with the exception of the thickness of the alveolar space, which we chose to limit to 8 μm . By choosing this thickness we are able to observe and quantify the contents of a single slice of the space that geometrically mimics alveoli without the need to use confocal microscopy, improving our imaging throughput. The thickness of these alveoli-inspired geometries allows the stacking of multiple *P. aeruginosa* and *S. aureus* cells, whereas *C. albicans* cells are limited to one (Fig. 1C, SI Fig. 1). In larger spaces with side length of 500 μm , a slowdown of growth emerges towards the closed end, indicating the development of nutritional gradients (SI Fig. 2A). Cells are loaded in the alveoli-inspired geometries at different species titers (Fig. 2D) and continuously perfused with fresh ASM for up to 20 hours while we perform timelapse microscopy. The observation window is limited to this interval because at later stages robust biofilm growth in the main channel starts influencing cell behavior in the alveoli-inspired geometries (SI Fig. 3). Across nine independent experiments (Fig. 2D) and 900 alveoli-inspired geometries, the robust emergence of spatial organization is observed (Fig. 2E and 2G) with a tendency of *C. albicans* to localize towards the closed end and the edges (SI Video 1). This displaces *P. aeruginosa* and *S. aureus*, which tend to localize towards the center and the entrance of the box. The displacement is most evident in *S. aureus* (Fig. 2H). This spatial organization emerges in less than 10 hours and remains stable throughout the course of the observations (Fig. 2I). As expected, the outcome has a certain dependence on the seeding titer of the species, with high *C. albicans*-to-bacteria ratios leading to dominance by the fungus and vice versa (Fig. 2D). To ensure that fluorescence measurements in the geometrical alveoli mimics are a good proxy for the biomass of each species, we carry out control experiments inside species-specific mother machines (SI Fig. 4). These allow us to simultaneously extract biomass from the z-limited cross-area of the cells and their fluorescence across different growth stages. Because the two quantities are in good agreement, we utilize the time traces of each fluorescence channel to extract the growth rate of each species in the polymicrobial context within the alveoli-inspired geometries. Despite the observed sharing of space and the robust tendency of *C. albicans* to

conquer the closed end of the alveoli-inspired geometries, we find significantly different growth rates, with the fungus growing the slowest (SI Fig. 5).

Spatial organization is robust to the presence of a clinically relevant *P. aeruginosa mexT* mutant

As a first test of the robustness of the emerging spatial organization against changes in the virulence of its members, we replaced the *P. aeruginosa* PA01 reference strain with a *mexT* frameshift mutant spontaneously emerged in the same background. Inactivation of *mexT* is a common feature in clinical isolates, leading to increased expression of virulence factors such as motility and pigments secretion [72]. The *mexT* mutant tends to form large rough colonies, which express significant amounts of a green pigment called pyocyanin [88] (Fig. 3A). Microscopy of the colonies on ASM agarose surfaces shows increased fitness of the mutant compared to the already dominant reference strain (Fig. 3B, 3C and 3D). *mexT* polymicrobial colonies grow larger (Fig. 3C), with *C. albicans* outcompeted even when 100 times more abundant than *P. aeruginosa* and *S. aureus* at the seeding (Fig. 3B and 3D). In the geometrical alveoli mimics with the *mexT* mutant, seeding communities at different species titers (Fig. 3E), again, shows that spatial organization emerges robustly across 6 independent experiments and 600 alveoli (Fig. 3F and 3H). In this case, the exclusion of *P. aeruginosa* from the closed end is less severe, but more severe for *S. aureus* (Fig. 3I). Although the mutant has a slightly higher growth rate than the reference strain (Fig. 3G and SI Fig. 4C), spatial organization still emerges within 10 hours (Fig. 3L, SI video 2) and at the same seeding titers (Fig. 3E).

Species composition influences spatial organization

Having characterized the behavior of a 3-species community, we investigate the contributions of single members by studying pair interactions. When grown in the absence of *C. albicans*, *S. aureus* and the *P. aeruginosa* reference strain tend to share the niche (Fig. 4, SI video 3). The *mexT* mutation alters this balance, resulting in a characteristic succession pattern in which *S. aureus* initially grows robustly to then be removed from the box by *P. aeruginosa* that systematically conquers the edges (Fig. 4A, second row, SI Video 4). In the presence of *C. albicans*, the absence of either *P. aeruginosa* (SI Video 5) or *S. aureus* (SI Video 6) shows little impact on spatial organization, with the yeast preferentially occupying the edges and the closed end of the alveoli-inspired geometries after 8-12 hours as in the three-species scenario (Fig. 4). Similar to the *P. aeruginosa*-*S. aureus* pair, *mexT* shifts the balance towards *P. aeruginosa* (Fig. 4, SI Video 7).

***C. albicans* conquers the closed end of confinements by growing radially with hyphae**

Our results show that *C. albicans* tends to occupy the edges of confinements and the closed end of our alveoli-inspired geometries. This could be the main

322 driver of spatial organization. We therefore seek to investigate how the slowest
 323 growing species can have such a dominant role in the ecology of a pathogenic
 324 polymicrobial community. Although *C. albicans*'s biomass grows overall more
 325 slowly in the alveoli-inspired geometries, its transition to hyphae in ASM focuses
 326 all growth in radial directions by virtue of the long asymmetric morphology of
 327 these structures (Fig. 5A). Although growth on a surface is known to stimulate
 328 hyphal transition, the ASM used here possesses all the necessary and suffi-
 329 cient conditions to induce hyphal morphogenesis (SI Fig. 6). In confinement,
 330 this allows it to rapidly reach the edges of the alveoli-inspired geometries and
 331 align along the walls. To test whether hyphal growth is necessary for spatial
 332 organization, we repeated polymicrobial experiments using a yeast-locked *C.*
 333 *albicans* strain that does not form hyphae, as it lacks the HGC1 gene, encoding
 334 a cyclin-related protein necessary for hyphal transition in ASM (Fig. 5B). This
 335 abolishes the spatial organization observed with the hyphae-competent strain
 336 (Fig. 5C, 5D, 5F and 5G, SI Video 8). However, the *hgc1Δ:Δ* strain has a slower
 337 growth rate than the reference strain (SI Fig. 5).

338 To further test whether the radial growth conferred to *C. albicans* by its
 339 hyphae determines spatial organization, we simulate a mutant with an identical
 340 growth rate *in silico*, using 2D agent-based simulations. A 2D alveoli-inspired
 341 geometry is populated by two species with growth rates and cell sizes that
 342 match those observed in experiments for the reference strain *C. albicans* and
 343 bacteria (grouping together *P. aeruginosa* and *S. aureus*, see *Methods* section).
 344 A single cell of each is initialized in the middle of the geometrical alveoli mimic.
 345 Strikingly, the hyphal *C. albicans* reside at the edges of the geometrical alveoli
 346 mimic, reproducing the spatial organization observed in experiments (Fig. 5H,
 347 SI Video 9). To directly test the role of radial *C. albicans* growth, we switch the
 348 reference *C. albicans* with a *hgc1Δ:Δ*-like strain that does not form elongated
 349 cells but has the same growth rate (Fig. 5I). In this case, the species mix without
 350 producing spatial organization (SI Video 10). To further confirm this is due to
 351 hyphae and not to the different single cell sizes, we simulate growth of hyphal *C.*
 352 *albicans* and yeast-locked mutant within the same niche at identical growth rate
 353 and again observe the localization of the cells that form hyphae at the periphery
 354 (Fig. 5J, SI Video 11).

355 Our simulations (Fig. 5) are initialized with both species starting with the
 356 same number of cells (one) at the centre of the niche; however relative numbers
 357 and starting positions are expected to impact the efficacy of radial growth for
 358 establishing and maintaining spatial structure. Simulations that start with large
 359 numbers of CA and bacterial cells homogeneously spread throughout the niche
 360 exhibit less pronounced spatial structure (SI Fig. 7). These predict that spatial
 361 organization emerges at intermediate times before eventual bacterial dominance.
 362 A certain dependence on relative numbers is observed also in the experiments
 363 (Fig. 2D and 3E). These results demonstrate that spatial structure within the
 364 geometrical alveoli mimics is driven by the asymmetrical growth of *C. albicans*
 365 hyphae, which, by concentrating in one direction, allows them to extend to the
 366 walls and corners of the mimic, though initial cell position and numbers could
 367 negate this advantage. Because the simulations only consider growth rates and

368 mechanical interactions, they suggest that *C. albicans*' dimorphism is sufficient
 369 to induce spatial organization. Although other mechanical and biochemical
 370 interactions are possible, they are not necessary to recapitulate the observed
 371 spatial organization and coexistence.

372 Discussion

373 By comparing agarose surfaces and confining microfluidic alveoli-inspired ge-
 374 ometries, we have shown that spatially structured niches can alter ecological out-
 375 comes in pathogenic microbes. The quasi-2D confinements reveal that, within
 376 10 hours after seeding, a polymicrobial community of *P. aeruginosa*, *S. aureus*,
 377 and *C. albicans* acquires and maintains a characteristic spatial organization
 378 that is not achieved on unconfined planar surfaces. The pivotal factor in this
 379 behavior is the capability of *C. albicans* to line the edges of the confined space
 380 with its hyphae, limiting the presence of the other members of the community.
 381 This strategy allows the fungus to survive in the niche despite its slow growth
 382 rate, coexisting with the faster growing *S. aureus* and *P. aeruginosa*. In con-
 383 trast, in the absence of spatial limits on a planar surface, *P. aeruginosa* quickly
 384 outcompetes *C. albicans*, even when starting from a hundred-fold numerical
 385 disadvantage. Like in real alveoli, the edges of the alveoli-inspired geometries
 386 mediate the contacts between microbes and substrate. In real alveoli, this con-
 387 tact is negotiated by the respiratory epithelium. Therefore, due to its tendency
 388 to line confined spaces, *C. albicans* may mediate most of the physical contact
 389 between the host and the polymicrobial community *in vivo*. This could have
 390 repercussions on treatment design, for example because targeting *C. albicans*
 391 might lead to its replacement with more pathogenic microbes. Instead, strate-
 392 gies that blunt *C. albicans*' toxicity without removing it from the niche may
 393 protect the host from further damage.

394 Our quasi-2D models are inspired by environments which would normally be
 395 3D and although the model enables us to specifically study the impact of edges
 396 and geometry, other factors such as nutrients gradients might become more
 397 important in 3D situations, where cells in the bulk might have more difficult
 398 access to these chemicals. Many other differences exist in comparison with
 399 pulmonary alveoli, including the fact that these do not normally tend to be
 400 flooded before infection. As in the case of edema, flooding can occur as a
 401 response to infection and tissue damage. Our model is therefore better suited
 402 to describe these conditions rather than the colonization of a sterile alveolar
 403 space. In the latter, microbes would start colonization already close to the edges
 404 and dynamics might be different. Although this will require further research,
 405 we expect the mechanisms observed in this work to still be relevant in these
 406 conditions, with hyphae still able to displace other microbes. Indeed, in the
 407 case of a thin liquid layer, surface tension would narrow down the range of
 408 possible orientations of hyphal tip growth, limiting it to those pointing towards
 409 the surface or parallel to it.

410 The robustness of the observed spatial organization and coexistence was

411 tested with a *P. aeruginosa mexT* mutant, an adaptation that is frequently
 412 found in clinical isolates [72]. *mexT* is a transcription regulator that controls
 413 the expression of more than 40 genes [89]. Its deletion increases virulence by
 414 upregulating motility and the production of secreted virulence factors, such as
 415 pyocyanin [88]. Although in the three-species experiments considered here, the
 416 mutant does not qualitatively alter the overall spatial organization trend, it does
 417 show increased ecological competitiveness. This is evident from a comparison
 418 between Fig. 2G and Fig. 3H with the mutant tending to a distribution that
 419 is skewed towards the edges of the microenvironments. Its increased ecolog-
 420 ical competitiveness manifests itself fully in two-species infections, where the
 421 *mexT* mutant is able to completely eradicate *S. aureus* and limit *C. albicans*'
 422 number (Fig. 4A). Because its growth rate is similar to that of the reference
 423 strain (SI Fig. 5, SI Fig. 4C), and those of *S. aureus* and *C. albicans* are not
 424 particularly affected by its presence, we hypothesize that its advantage is due to
 425 increased motility. This could allow the bacterium to make its way around *C.*
 426 *albicans*' biomass and dislodge *S. aureus* from crevices, pushing it away through
 427 growth. Although on the unconfined surface we found indications of competi-
 428 tive inhibition, likely due to the dynamics of nutrients usage, we did not observe
 429 them in the microenvironments which are continuously perfused. Other muta-
 430 tions that upregulate contact-dependent and independent toxin production both
 431 in the lung context and beyond might alter the balance we have observed.

432 As a mechanism for the emergence of spatial organization, we hypothesized
 433 that hyphae allow *C. albicans* to concentrate its growth in a single direction,
 434 enabling it to reach the edges of the confinement before being removed by the
 435 faster growing species. *In silico* experiments of single cells placed in direct
 436 competition, confirm this view. We further tested this hypothesis with a yeast-
 437 locked mutant that does not form hyphae, finding that it does not give rise
 438 to spatial organization or persist in the alveoli-inspired geometries. However,
 439 the mutant has a slower growth rate than the reference strain, and thus we
 440 repeated the experiment *in silico*, this time matching the growth rates. The
 441 simulations confirmed our hypothesis, indicating that an elongated morphology
 442 in a slower-growing species is sufficient to offset the ecological outcome in a
 443 confinement.

444 Taken together, our findings demonstrate that traits controlled at the level
 445 of phenotype, such as *C. albicans*' morphological changes and *P. aeruginosa*'s
 446 virulence, can drastically change ecological results when observed in realistic
 447 settings to the point of outweighing the growth rate. Because such evolutionary
 448 adaptations are prevalent among microbes and microscale spatial structure is
 449 widespread across different environments, we expect many other examples to
 450 exist in Nature.

451 Author Contributions

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 462 Writing – review and editing.

463 Acknowledgements

464 We thank Bartłomiej Waclaw, Teuta Pilizota and Mie Monti for insightful
 465 discussions. We are also thankful to Simon Foster, Rob Wheeler and Yue
 466 Wang, and Tim Tolker-Nielsen for the gift of the *S. aureus*, *C. albicans*, and
 467 *P.aeruginosa* strains, respectively. LM acknowledges funding from the Herchel
 468 Smith fund, Postdoctoral fellowship. JK and PC acknowledge funding from the
 469 UK CF Trust SRC 016. EB acknowledges funding from the Oliver Gatty Trust.
 470 MW was supported by the CF Trust (a Venture and Innovation Award (VIA))
 471 and from the Leverhulme Trust. This research has received funding from the
 472 European Research Council (ERC) under the European Union’s Horizon 2020
 473 research and innovation program (Grant agreement No. 851196). For the pur-
 474 pose of open access, the author has applied a Creative Commons Attribution
 475 (CC BY) license to any Author Accepted Manuscript version arising from this
 476 submission.

477 Competing interest

478 None declared.

479 Data and materials availability statement

480 Data are available at 10.5281/zenodo.15005092. Scripts used for analysis are
 481 available at
 482 <https://github.com/mlaenoc/alveoli-code>. Code for the simulations is available
 483 at
 484 <https://github.com/roryclaydon1994/BiofilmDES>.

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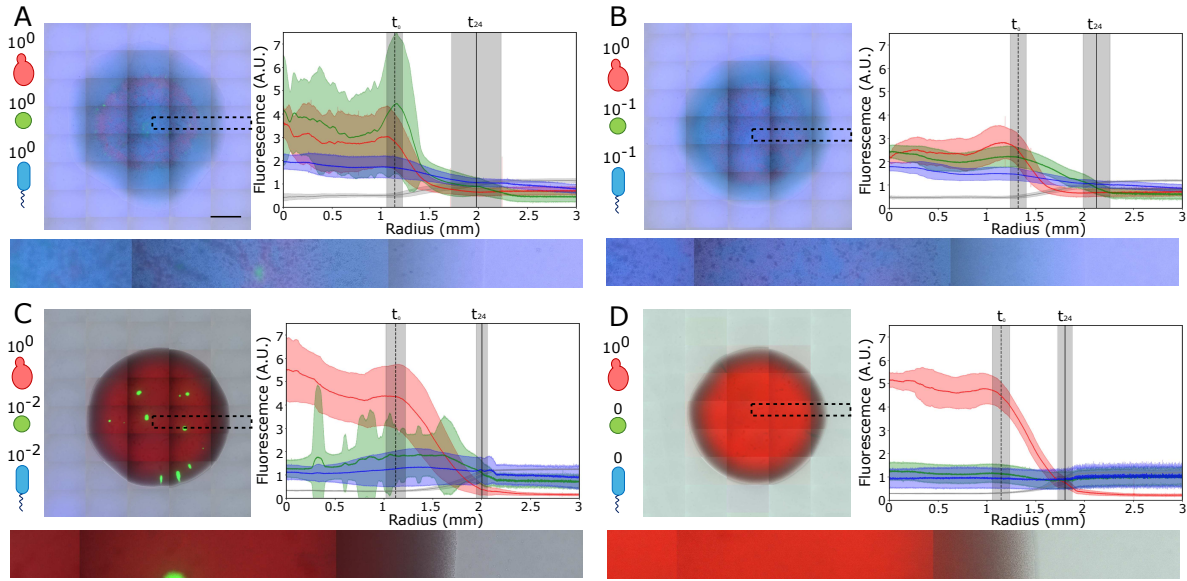


Figure 1: Quantification of the growth of a PA-SA-CA community on an unlimited surface. a) PA-SA-CA polymicrobial community seeded at a 1:1:1 ratio on soft agar. (left) example colony after 24 hours growth at 37°C. (bottom) zoom-in of the highlighted area. (right) average intensity profiles for the red, green, blue (fluorescence) and gray (brightfield) channels. Gray reports brightfield intensity and is a proxy for total colony size. To obtain the traces, colonies are split in 360 sections, each 1° large. Radii between the centroid and the edges of the image are drawn and averaged. The shaded areas show the standard deviation of all radii across 5 colonies. The vertical black lines indicate the average radius of the colony at the start and after 24 hours. The shaded area shows the standard deviation. Scale bar = 1 mm. b) PA-SA-CA polymicrobial community seeded at a 0.1:0.1:1 ratio. Averages and standard deviations of 3 colonies. c) b) PA-SA-CA polymicrobial community seeded at a 0.01:0.01:1 ratio. Averages and standard deviations of 3 colonies. d) Growth of a colony of *C. albicans* in isolation. Averages and standard deviations of 3 colonies. Epifluorescence microscopy, stitched FOVs. The bottom strips are zoom-ins of the areas highlighted by the dotted lines. Channel-separated, grayscale versions of this figure are given at the end of SI.

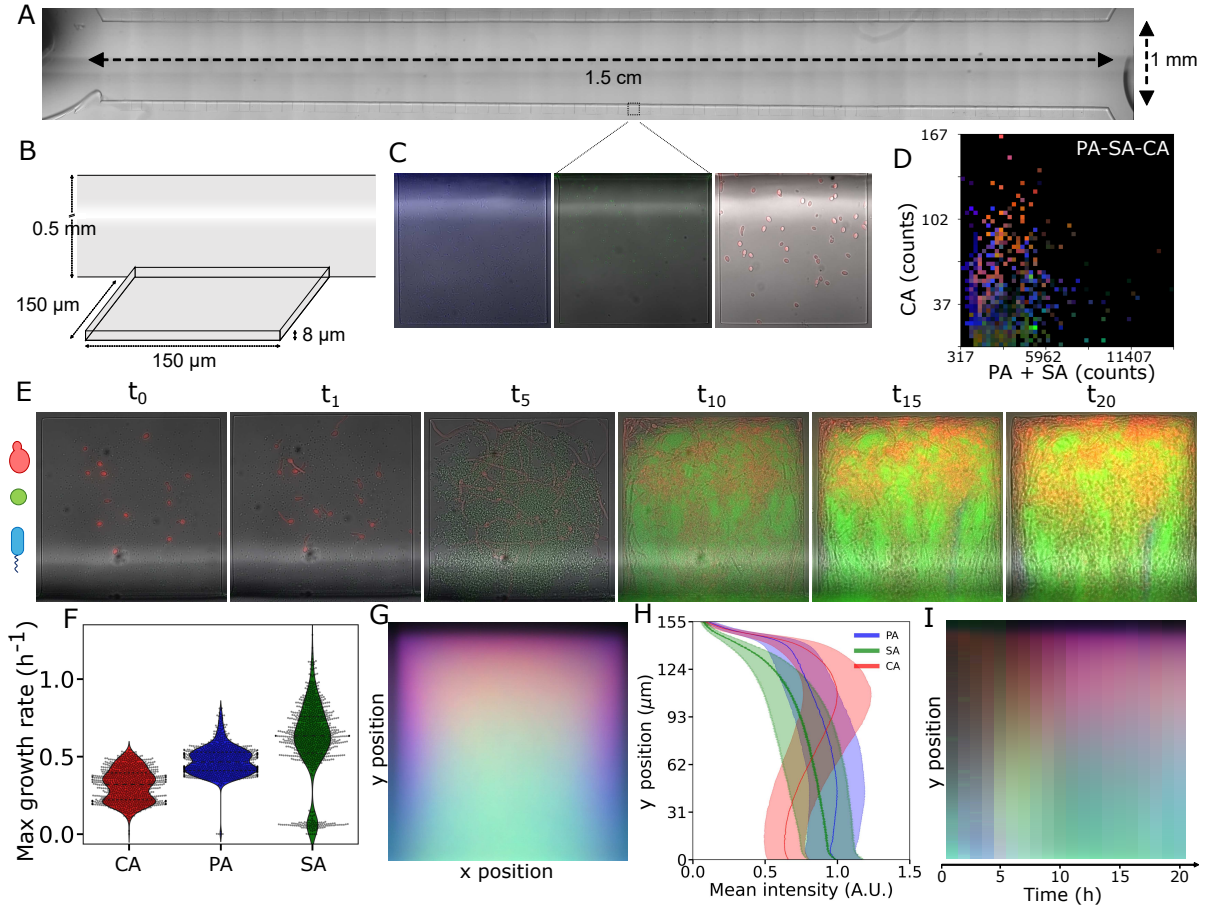


Figure 2: Quantification of the growth dynamics of a PA-SA-CA community in spatially structured environments. a) architecture and dimensions of the microfluidic chip. b) zoom-in to the dimensions of the quasi-2D alveoli-inspired geometries. c) *P. aeruginosa* (left, blue), *S. aureus* (center, green), and *C. albicans* (right, red) at the seeding. They express eCFP, eGFP and dTomato, respectively. d) mean fluorescence for dTomato, eGFP and eCFP expressed as RGB colour in the distal quarter (closed end) of the microchambers after 20 hours, plotted as a function of the initial seeding conditions (number of yeast cells against number of bacterial cells). e) example time traces from different experiments of the PA-SA-CA community growing in the alveoli-inspired geometries. f) maximum growth rates extracted from the time course of the different fluorescence channels. P values obtained with Welch's t-test are all $< 1e - 47$. g) average behavior at 20 hours, obtained by summing up all of the boxes. h) average and standard deviation of the fluorescence intensity of the three fluorescence channels, proxy for PA, SA, and CA abundance, along the vertical axis of (g). i) kymograph showing the progression of the average behavior over 20 hours of observation. Each band is obtained by averaging the mean image (g) along the x axis. In all cases, the side length of the square alveolar geometries is 150 μm . In all cases, the figures are generated from data from 9 independent experiments with 100 replicates (boxes) each. Channel-separated, grayscale versions of this figure are given at the end of SI.

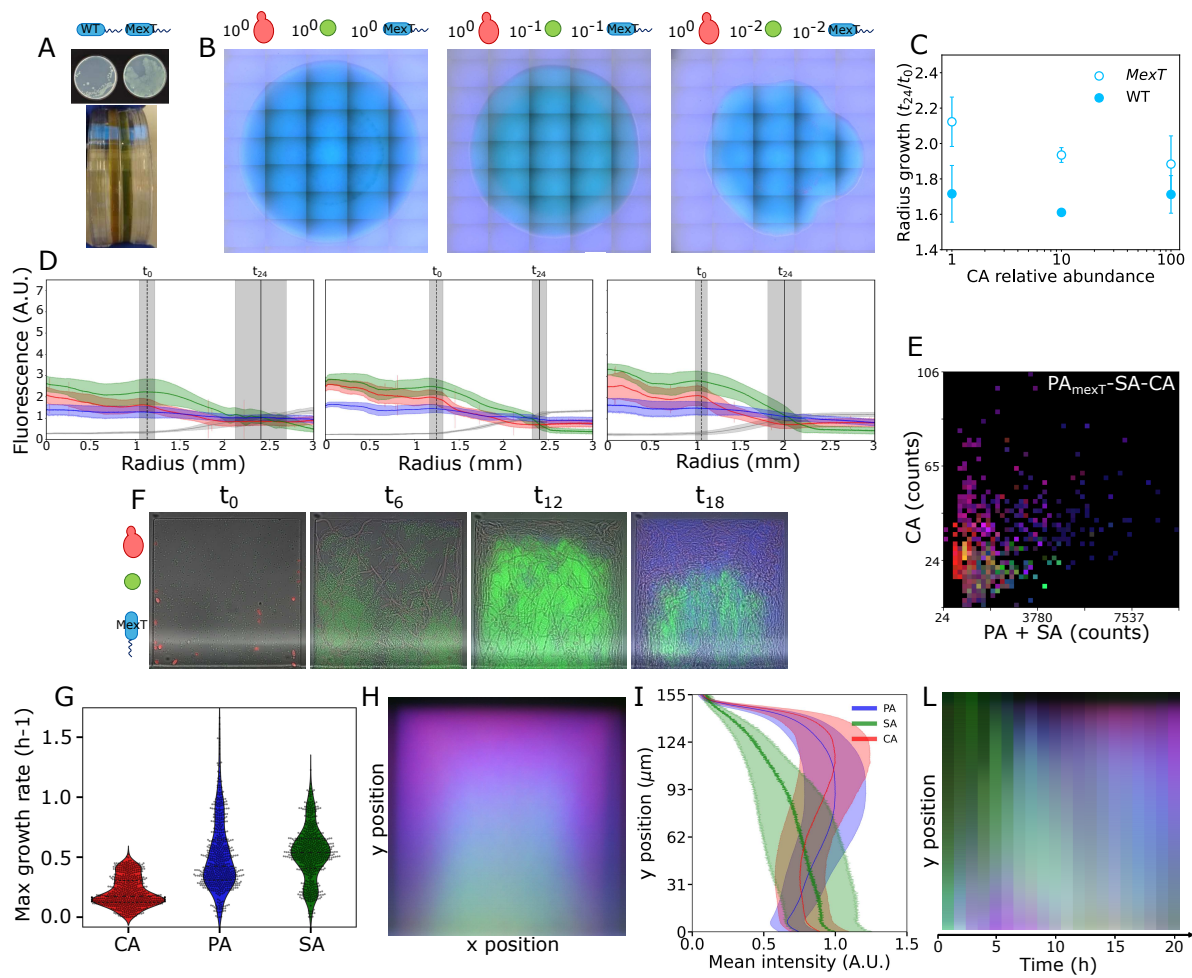


Figure 3: Test of the robustness of the spatial organization of a PA-SA-CA community in which *P. aeruginosa* carries a clinically relevant *mexT*-inactivating mutation. a) the *mexT* mutant shows a characteristically enlarged colony size (top) and secretes large quantities of piocyanin (bottom). b) PA_{*mexT*}-SA-CA polymicrobial community seeded at various ratios (left 1:1:1, center 0.1,0.1,1, right 0.01,0.01,1). The *P. aeruginosa* strain expresses the mutated *mexT*. Scale bar = 1 mm. c) comparison of radius growth between PA-SA-CA communities with a reference strain of *P. aeruginosa* (filled circles) or the *mexT* mutant (empty circles). The error bar shows the standard deviation between repeats (6 colonies for a PA-SA-CA initial titer of 1:1:1, 3 colonies for 0.1,0.1,1, and 6 colonies for 0.01,0.01,1. P values for 1, 10 and 100 CA relative abundance are: 0.004, 0.004 and 0.15, respectively. d) average intensity profiles for red, green, blue (fluorescence) and gray (brightfield) channels. Gray indicates brightfield intensity as a proxy for total colony size. Traces are obtained as the average of 360 radii, one every angular degree of the colony. The shaded areas show the standard deviation. The vertical black lines indicate the average radius of the colony at the start and after 24 hours. The shaded area shows the standard deviation. (left) PA-SA-CA ratios 1:1:1, center (0.1,0.1,1), right (0.01,0.01,1). e) mean fluorescence for dTomato, eGFP and eCFP expressed as RGB colour in the distal quarter (closed end) of the microchambers after 20 hours, plotted as a function of the initial seeding conditions (number of yeast cells against number of bacterial cells). f) example time trace of the 3-species polymicrobial community containing the *mexT* mutant in the geometrical alveoli mimics. g) growth rates extracted from the timelines of the fluorescence channels. h) average behavior at 20 hours, obtained by summing up all of the boxes. i) average and standard deviation of the fluorescence intensity of the three fluorescence channels, proxy for PA, SA, and CA abundance, along the vertical axis of (h). l) kymograph showing the progression of the average behavior over 20 hours of observation. Each band is obtained by averaging the mean image (h) along the x axis. In all cases, the side length of the square alveolar geometries is 150 μm and the figures are generated from data from 6 independent experiments with 100 replicates (boxes) each. Channel-separated, grayscale versions of this figure are given at the end of SI.

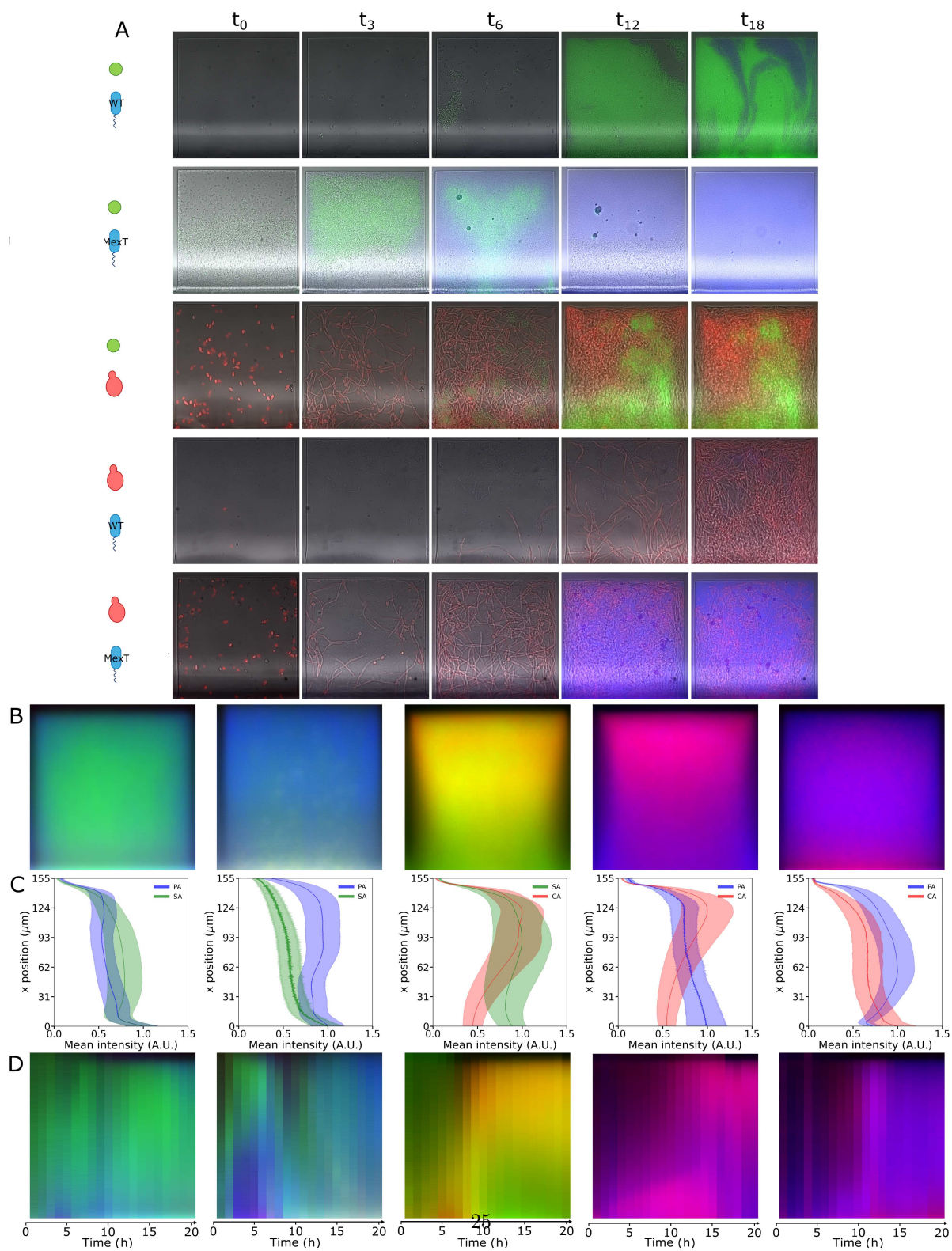


Figure 4: **Analysis of the role of each species in the determination of spatial organization.** a) example time series of PA-SA, PA_{mexT}-SA, SA-CA, PA-CA and PA_{mexT}-CA communities. b) average behavior at 20 hours, obtained by summing up all of the boxes (3 experiments, 300 boxes each). Spatial organization emerges also in the absence of SA or PA, but not if PA has a mutated *mexT*. c) average and standard deviation of the fluorescence intensity of the three fluorescence channels, proxy for PA, SA, and CA abundance, along the vertical axis of (b). d) kymograph showing the progression of the average behavior over 20 hours of observation. Each band is obtained by averaging the mean image (b) along the x axis. In all cases, the side length of the square alveolar geometries is 150 μm and the figures are generated from data from at least 3 independent experiments with 100 replicates (boxes) each. Channel-separated, grayscale versions of this figure are given at the end of SI.

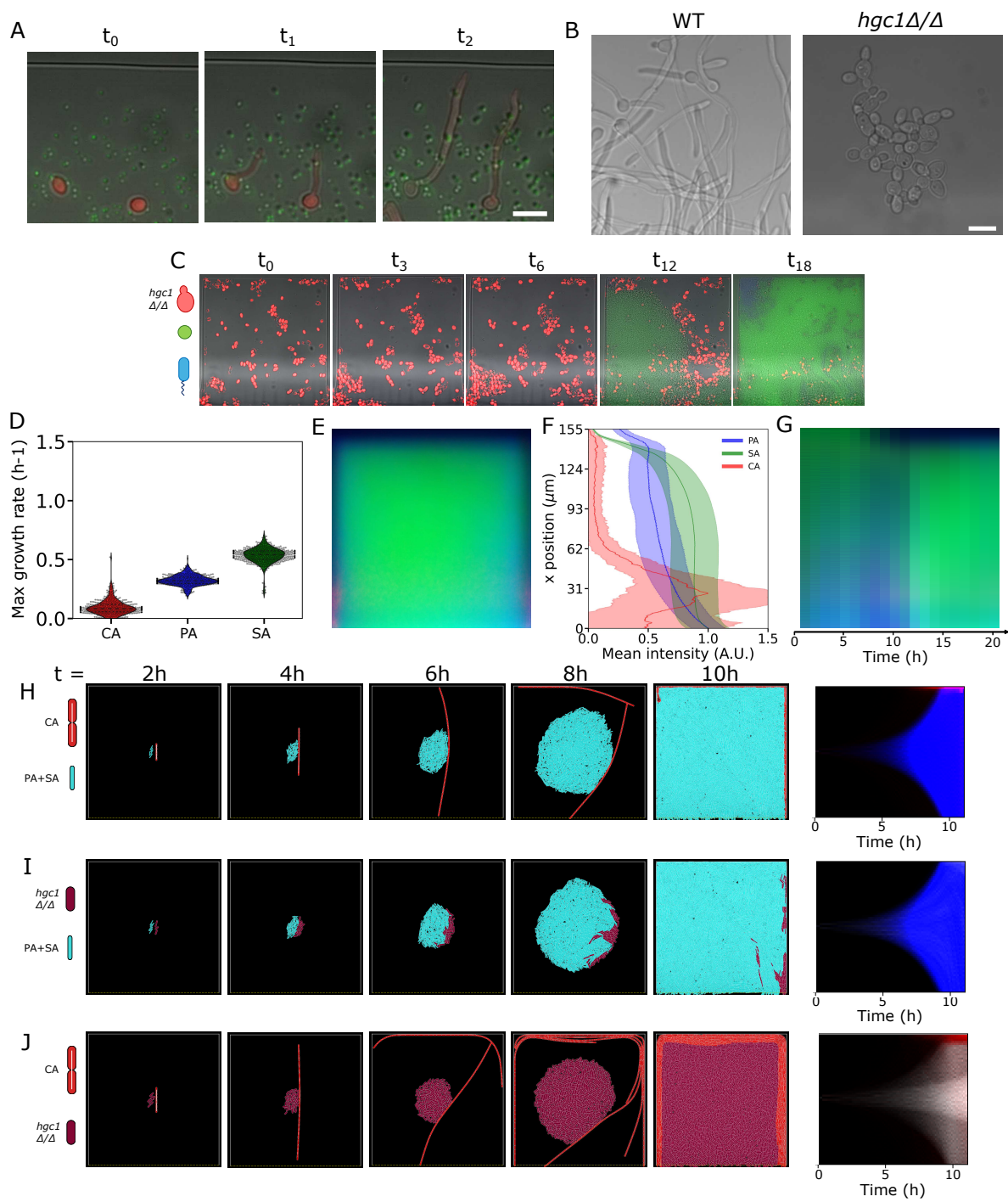


Figure 5: Analysis of the role of hyphal growth in the determination of spatial organization and coexistence. a) Representative transition from yeast to hyphal form of *C. albicans* within geometrical alveoli mimics. b) The yeast-locked mutant *hgc1* Δ : Δ does not grow hyphae in our conditions. c) example time lapse of the *hgc1* Δ : Δ growing in the presence of *P. aeruginosa* and *S. aureus*. *C. albicans* is segmented from brightfield and false coloured as explained in the methods section. d) growth rate of the 3 species grown in the geometrical alveoli mimics. e) average behavior at 20 hours, obtained by summing up all of the boxes (5 experiments, 500 boxes each). f) average and standard deviation of the fluorescence intensity of the three fluorescence channels, proxy for PA, SA, and CA abundance, along the vertical axis of (e). g) kymograph showing the progression of the average behavior over 20 hours of observation. Each band is obtained by averaging the mean image (e) along the x axis. h-j) 2D agent simulations initialized with 2 cells at the centre of the trap showing: h) that hyphae-competent *C. albicans* positions itself at the edges; i) that the yeast-locked mutant *hgc1* Δ : Δ does not enable spatial organization, even when it grows at the same growth rate of the reference strain; j) that a hyphae-competent species occupies the edges of confinement in the presence of a species that grows at the same rate, but that cannot form hyphae. At the end of each row, kymographs summarizing the dynamic progression of the simulations across at least 9 repeats are given. In the kymographs, the *hgc1* Δ : Δ is given in white. Scale bars = 10 μ m. In all cases, the side length of the square alveolar geometries is 150 μ m. Channel-separated, grayscale versions of this figure are given at the end of SI.