scientific reports

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# Impact of mixed *Staphylococcus aureus-Pseudomonas aeruginosa* biofilm on susceptibility to antimicrobial treatments in a 3D in vitro model

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***Staphylococcus aureus*** **and** ***Pseudomonas aeruginosa*** **are the most common bacteria co-isolated from chronic infected wounds. Their interactions remain unclear but this coexistence is beneficial for both bacteria and may lead to resistance to antimicrobial treatments. Besides, developing an in vitro model where this coexistence is recreated remains challenging, making difficult their study. The aim of this work was to develop a reliable polymicrobial in vitro model of both species to further understand their interrelationships and the effects of different antimicrobials in coculture. In this work, bioluminescent and fluorescent bacteria were used to evaluate the activity of two antiseptics (chlorhexidine and thymol) against these bacteria planktonically grown, or when forming single and mixed biofilms. At the doses tested (0.4-1,000 mg/L), thymol showed selective antimicrobial action against** ***S. aureus*** **in planktonic and biofilm states, in contrast with chlorhexidine which exerted antimicrobial effects against both bacteria. Furthermore, the initial conditions for both bacteria in the co-culture determined the antimicrobial outcome, showing that** ***P. aeruginosa*** **impaired the proliferation and metabolism of** ***S. aureus*****. Moreover,** ***S. aureus*** **showed an increased tolerance against antiseptic treatments when co-cultured, attributed to the formation of a thicker mixed biofilm compared to those obtained when monocultured, and also, by the reduction of** ***S. aureus*** **metabolic activity induced by diffusible molecules produced by** ***P. aeruginosa*****. This work underlines the relevance of polymicrobial populations and their crosstalk and microenvironment in the search of disruptive and effective treatments for polymicrobial biofilms.**

**Keywords** *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Polymicrobial biofilm, Antiseptics, Chlorhexidine, Thymol

The importance of pathogenic polymicrobial infections is becoming increasingly recognized, particularly in the context of biofilm formation, where various bacterial species interact synergistically or antagonistically competing for resources. In this context, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two commonly co-isolated bacteria in pulmonary infections and on chronic topical wounds[1]. The interactions between these two bacteria in co-infected tissues have been the focus of continuous research, as their coexistence contributes to enhanced virulence. It has been reported that wounds infected with both species typically exhibit delayed closure compared to wounds infected with a single species[2]. Alongside factors related to the immune response by the host, this delayed wound closure can be attributed to the increased expression of *S. aureus* virulence factors during co-infection, a pattern reported for the methicillin-resistant *S. aureus* (MRSA) strain USA300[2].

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There is no consensus on whether these bacteria have an antagonistic or mutualist relationship in complex physiological media. Some studies indicate that early colonisation by *P. aeruginosa* shows strong antagonism towards *S. aureus*[3], through the secretion of a wide variety of anti-staphylococcal molecules and proteases that inhibit *S. aureus* growth and proliferation[4,5]. This process induces a metabolic transition of *S. aureus* from aerobic respiration to fermentation and eventually leads to a reduction in *S. aureus* viability. In response to this hostile environment, *S. aureus* may adapt to *P. aeruginosa* virulence factors by increasing biofilm formation and by the presence of small colony variants inside infected eukaryotic cells[4]. In return, this would provide *S. aureus* with a greater capacity to withstand antimicrobial therapies. Additionally, exoproducts from *P. aeruginosa* have been shown to promote the production of staphyloxanthin, further enhancing the virulence of *S. aureus*[67]. Other studies have shown that *S. aureus* supports colonisation and pathogenicity of *P. aeruginosa* by inhibiting its phagocytosis by eukaryotic cells[8]. It has been proposed that, during the course of chronic infection, *P. aeruginosa* may find evolutionarily favourable to maintain a population of *S. aureus* to help counteract the host's immune response. Recent studies show that *P. aeruginosa* populations thrive in the presence of toxin-producing *S. aureus* strains in both lung and wound infections[2,9]. Moreover, mutant strains of *Pseudomonas* that have reduced anti-staphylococcal capacity are commonly isolated in chronic infections of patients with cystic fibrosis[10].

In multicellular communities, collective microbial dynamics generate a complex ecosystem characterised by both microbe-microbe and microbe-environment interactions. These features must somehow be present in in vitro models, but they are barely reproducible in planktonic cultures. Co-cultures of *S. aureus* and *P. aeruginosa* have been performed using modified media in static in vitro conditions, anoxia, microtiter plates, Calgary biofilm devices or even directly on mammalian cells[11–13]. Nevertheless, the development of an experimental model to recreate this coexistence proves to be a challenging task, as the in vitro interaction between both species appears to be antagonistic at first. Interestingly, while *P. aeruginosa* strongly inhibited in vitro the growth of *S. aureus*, this effect was considerably less pronounced in vivo[2]. This matter adds complexity to the assessment of the effectiveness of antimicrobial treatments against these polymicrobial communities.

A deeper understanding of the behaviour of co-cultures of these opportunistic bacteria is of extreme importance, not only because of their mutual impact on the bacterial behaviour and metabolic activity, but also because some studies have shown that their interplay contributes to antimicrobial tolerance. Notably, it has been reported that when *S. aureus* is able to coexist with *P. aeruginosa* in a co-culture, its tolerance to antibiotics significantly increases[11,14]. Conversely, there have also been studies indicating that *P. aeruginosa* can reduce the susceptibility of *S. aureus* to antibiotic treatment through mechanisms such as rhamnolipid production, HQNO, and LasA[15,16].

In this study, a reliable in vitro model of a polymicrobial biofilm of *S. aureus* and *P. aeruginosa* was successfully established to understand the interrelationships and crosstalk between both species and the effects of different antimicrobials under these conditions. The use of bioluminescent and fluorescent strains of both species was proposed to study optically the growth kinetics and metabolic state of these bacteria over time. It was investigated how the initial conditions of both bacterial species influenced their final interaction within the co-culture. Then, the antimicrobial activity of thymol (THY) and chlorhexidine (CHXD) was compared against both species in their planktonic state, as well as when forming single and polymicrobial biofilms. This comparison aimed to determine whether bacteria growth may change in polymicrobial biofilms and to help in the identification of improved antimicrobial treatments using an in vitro model that mimics the polymicrobial nature of human-relevant biofilms.

## Results and discussion

### Establishment of *S. aureus - P. aeruginosa* mixed biofilm

Two different strategies were assessed for developing an in vitro model of a polymicrobial biofilm containing both *S. aureus* and *P. aeruginosa* (Fig. 1). Using bioluminescent strains together with wild-type strains, the bioluminescence of both species was monitored over a 48 h period in parallel experiments. In both cases, the *P. aeruginosa* strains were added at progressively increased concentrations whereas *S. aureus* strains were always added at $10^7$ colony forming units (CFU)/mL.

In the first approach, *S. aureus* and *P. aeruginosa* were mixed as planktonic co-cultures. The normalised kinetics of the bioluminescent strains, as well as the bacterial count in the culture at the end of the experiments, are represented in Fig. 1.

Bioluminescence kinetics of *S. aureus* Xen36 exhibited a concentration-dependent suppression when co-cultured with *P. aeruginosa* PAO1, being ultimately reduced to levels close to the background signal (Fig. 1a). This observation suggested a substantial influence of *P. aeruginosa* PAO1 presence on *S. aureus* Xen36 bioluminescence which is directly related to its metabolic activity. Conversely, the bioluminescence signal of *P. aeruginosa* Lux remained unaltered under the presence of non-bioluminescent planktonic *S. aureus* 29213, depending solely on its initial concentration (Fig. 1b).

The analysis of the bacterial counts in the culture at the end of the experiment (Fig. 1c) aimed to assess whether the decrease in bioluminescence resulted from a reduction in bacterial numbers, from a decline in the metabolic activity, or from a combination of both factors. For *P. aeruginosa* concentrations ranging from $10^2$ to $10^4$ CFU/mL, *S. aureus* growth was slightly inhibited, as its concentration was arrested around 2 logs ($10^7$ CFU/mL) compared to that of the control sample. For *P. aeruginosa* concentrations between $10^5$ and $10^8$ CFU/mL, *S. aureus* concentration was significantly reduced, resulting in a decrease of $10^6$ CFU/mL compared to that of the control sample. Because no substrate limitation was present, *S. aureus* growth inhibition may be attributed to the presence of anti-staphylococcal compounds released by *P. aeruginosa*. In this sense, previous studies have also revealed the prevalence of *P. aeruginosa* in planktonic cultures even using different culture media[11] or clinical strains[3].

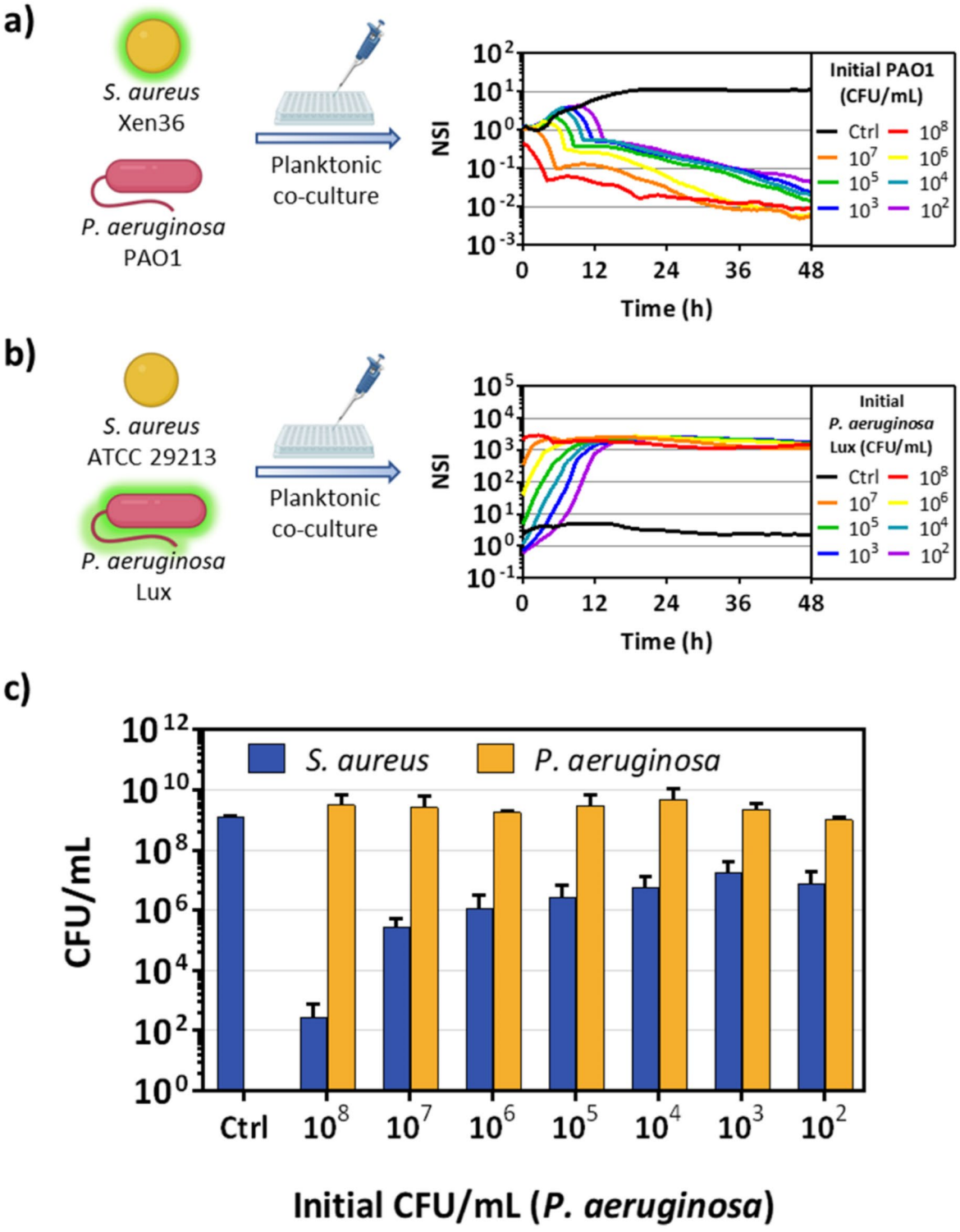


**Fig. 1**. Bioluminescence signals from **(a)** Planktonic *S. aureus* Xen36 ($10^7$ CFU/mL) co-cultured with increasing amounts of planktonic *P. aeruginosa* PAO1 ($10^1$-$10^8$ CFU/mL) and **(b)** Planktonic *P. aeruginosa* Lux ($10^1$-$10^8$ CFU/mL) co-cultured with a fixed amount of *S. aureus* ATCC 29213 ($10^7$ CFU/mL). The acronym NSI stands for "Normalized Signal Intensity". Control samples (Ctrl) represent the normal growth of cultures of *S. aureus* without the addition of *P. aeruginosa*. **(c)** Bacterial counts of both species in the cultures after 48 h (data obtained with conditions (**a**) and (**b**) were similar). The data are represented as Mean ± SD of three independent experiments.

In the second approach, the different strains of *S. aureus* were first allowed to grow into a 48-hour-old mature biofilm. Subsequently, a co-culture was established by adding increasing concentrations of planktonic *P. aeruginosa.* Figure 2 displays both the bioluminescence of the strains and the final bacterial counts. Unlike the first approach, where the relative bioluminescence measured at 48 h decreased with increasing *P. aeruginosa* concentrations, a constant low level of relative bioluminescence (a 2.5 log reduction compared to pure *S. aureus* biofilm) was observed regardless of the initial *P. aeruginosa* concentration (Fig. 2a and b). Nevertheless, when *S. aureus* Xen36 was already forming a mature biofilm, the presence of planktonic *P. aeruginosa* did not significantly inhibit its growth after 48 h (Fig. 2c). Despite a one-log reduction compared to the control, both species coexisted in the culture, indicating that *S. aureus* biofilm may provide some level of protection from the bactericidal and inhibitory effects caused by *P. aeruginosa* compared to the results observed in their planktonic state. It has been widely reported that when *P. aeruginosa* and *S. aureus* are grown together in co-culture, *P. aeruginosa* becomes dominant, outcompeting, and outgrowing *S. aureus*[16]. Herein, we demonstrate that under co-culture conditions the growth of one species depends on the concentration of the other and on its status (sessile or planktonic). These findings are consistent with previous studies that have shown that when *S. aureus* serves as the pioneer colonizer in biofilm growth assays, it promotes the attachment of *P. aeruginosa*[17]. Additionally, these studies suggest that individuals pre-colonized with *S. aureus* are more vulnerable to secondary colonization by *P. aeruginosa*.

## Study of the interaction between *S. aureus* and *P. aeruginosa* in mixed biofilm

To explore if *P. aeruginosa* requires direct contact with *S. aureus* to hinder its growth, an experiment using Transwell® inserts was conducted, in which the bacterial species shared liquid media while avoiding physical contact. Figure 3a showcases an overview of this experiment, in addition to the measurement of the bioluminescent kinetics and bacteria counts after 48 h.

As observed in Fig. 3b, the bioluminescence signal of *S. aureus* Xen36 was only reduced after 24 h when it was in direct physical contact with *P. aeruginosa*. In cases where *P. aeruginosa* was grown in the insert, the signal remained at levels similar to those of the control culture. However, by the 48-hour mark, the signal in all cultures, except for the control, decreased to baseline levels. As depicted in Fig. 3c, the interaction with *P. aeruginosa* resulted in a slight reduction of approximately one logarithm in the growth of *S. aureus*, although this value is not statistically significant. These experiments suggest that the decrease in bioluminescence may be attributed to a suppression of their metabolic activity. It is also worth mentioning that the physical proximity of *P. aeruginosa* could potentially accelerate this process.

To determine whether *P. aeruginosa* requires the presence of *S. aureus* to secrete molecules that interfere with *S. aureus* metabolism, conditioned medium (CM), in which *P. aeruginosa* was grown alone, was added to a 48-hours-old *S. aureus* GFP biofilm. For these experiments, the bioluminescent strain (*S. aureus* Xen36) was substituted with the fluorescent strain (*S. aureus* GFP) considering its suitability for subsequent confocal microscopy studies. Unlike bioluminescence, which depends on both ATP and protein synthesis, fluorescence is solely reliant on GFP production, offering more specific insights into how *P. aeruginosa*-secreted molecules impact *S. aureus*. The GFP fluorescence was then monitored over a 70-hour period (Fig. 4).

When *S. aureus* GFP was not exposed to CM, GFP fluorescence intensity showed a steady increase over time, reaching a plateau after 18 h. Conversely, when *S. aureus* was exposed to CM, a rapid and substantial decrease in GFP fluorescence intensity was observed within the first hour, followed by an overall reduction in the maximum fluorescence intensity reached. This suppression effect on GFP fluorescence was found to be concentration-dependent, with a degree of suppression gradually decreased as the CM was diluted. These results suggest that *P. aeruginosa* PAO1 produced one or more diffusible molecules capable of suppressing both the luminescence and fluorescence of *S. aureus* biofilms in a concentration-dependent manner, without affecting bacterial viability, as evidenced by stable CFU/mL counts. The decrease in bioluminescence at a constant bacterial count may result from reduced ATP levels, oxygen availability, or protein synthesis. Meanwhile, the reduction in fluorescence likely points to the suppression of green fluorescent protein (GFP) synthesis. However, the potential impact on ATP levels or protein synthesis was not assessed in this study and should be evaluated to validate this hypothesis.

The anti-staphylococcal properties of *P. aeruginosa* were initially documented in the 1950s[18]. During this period, researchers identified 4-hydroxy-2-heptylquinoline N-oxide (HQNO) as the primary compound produced by *P. aeruginosa*. This compound effectively impeded the cytochrome systems of various bacteria, including *S. aureus*[19]. While HQNO is recognized as an anti-staphylococcal agent, it does not induce lysis in *S. aureus* but retards its growth by inhibiting its oxidative respiration and ATP production.

HQNO is produced exclusively under aerobic conditions in the presence of oxygen[20], suggesting that in the low-oxygen environments observed in thick *S. aureus* biofilms[21,22], HQNO may have a limited role in mediating the interference between *P. aeruginosa* and *S. aureus.* Other documented anti-staphylococcal agents secreted by *P. aeruginosa* include pyocyanin[4] and the LasA protease bacteriocin[23]. Considering our results, it is worth noting that *P. aeruginosa* may produce anti-staphylococcal agents regardless the environmental conditions. According to the literature, the number, quantity, and structure of these agents produced by *P. aeruginosa* can vary across different strains and growth conditions, such as planktonic versus biofilm states or in the presence of host factors and antibiotics. However, as attested in this work, these agents would only have mild bactericidal effectiveness against *S. aureus* once a mature biofilm is formed.

## Antimicrobial activity tests

In order to assess whether the polymicrobial biofilm provides any advantage to bacteria against antimicrobial action, two different antimicrobials were selected to evaluate their effects on planktonic bacteria, as well as on single and mixed biofilms. The mixed biofilms were prepared following the methodology previously outlined in Fig. 2a and b. CHXD (1,6-bis(4-chloro-phenylbiguanido)hexane) was chosen as an example of a synthetic

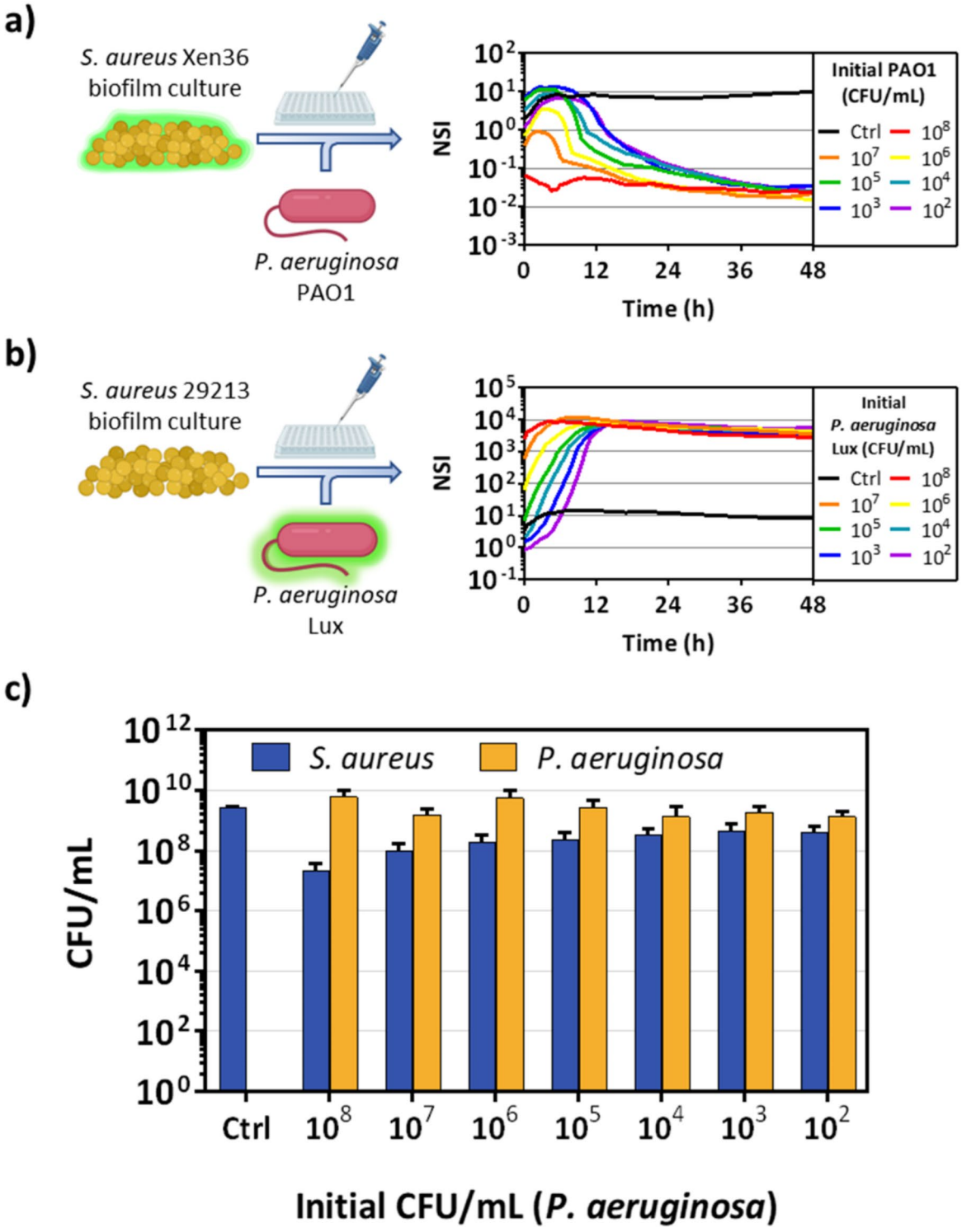


**Fig. 2**. Bioluminescence signals from **(a)** 48-hours old *S. aureus* Xen36 biofilm supplemented with increasing amounts of planktonic *P. aeruginosa* PAO1 ($10^1$-$10^8$ CFU/mL) or **(b)** 48-hours old *S. aureus ATCC 29,213* supplemented with increasing amounts of planktonic bioluminescent *P. aeruginosa* lux ($10^1$-$10^8$ CFU/mL). **(c)** Mean bacterial counts of both species in the cultures after 48 h (data obtained with conditions (**a**) and (**b**) were similar). The data are represented as Mean ± SD of three independent experiments.

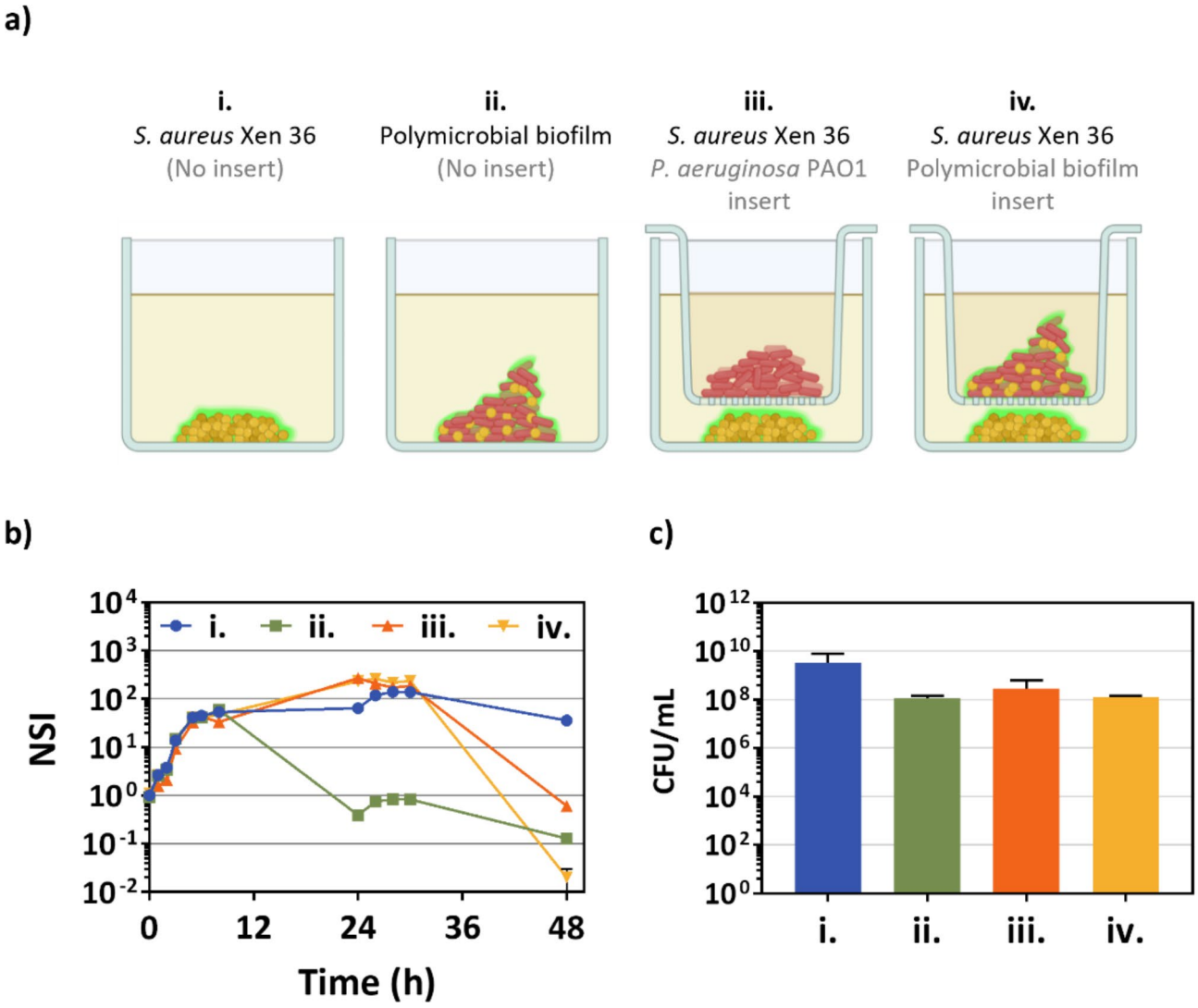


**Fig. 3**. Interaction between both species in mixed biofilm: **(a)** Schematic overview of the methodology developed to evaluate the interaction between bacterial species: **i.***S. aureus* Xen36 single biofilm culture without insert. **ii.***S. aureus* Xen36 and *P. aeruginosa* PAO1 mixed biofilm without insert. **iii.***S. aureus* Xen36 and *P. aeruginosa* single biofilm in the insert. **iv.***S. aureus* Xen36 single biofilm, and *S. aureus* Xen36 and *P. aeruginosa* PAO1 mixed biofilm in the insert. **(b)** Bioluminescence signal of *S. aureus* Xen36 over 48 h. **(c)***S. aureus* Xen36 counting measured after 48 h.

antiseptic commonly used in clinical settings whereas thymol (THY) (5-Methyl-2-(propan-2-yl)phenol) was chosen as an example of a natural origin antiseptic both used for skin disinfection. Results are represented in Fig. 5, while minimum biofilm inhibitory concentrations (MBIC) and minimum biofilm eradication concentrations (MBEC) data are summarized in Table 1.

In this context, it was observed that, in the range of concentrations tested, CHXD effectively eliminated both bacterial species, with the exception of *P. aeruginosa* in its biofilm and mixed biofilm forms, as well as *S. aureus* in mixed biofilm. Notably, in the case of *S. aureus* within the mixed biofilm, its MBIC against CHXD also increased by a factor of 2.5 when compared with that obtained in its single biofilm form. On the other hand, THY displayed selective antimicrobial activity against *S. aureus*, not observing bactericidal activity against *P. aeruginosa* at the doses tested. THY successfully eradicated *S. aureus* in its planktonic state at concentrations above 600 mg/L, but it was less effective when forming either single or mixed biofilms. In the latter scenario, the MBIC of THY increased from 700 to 1000 mg/L when compared with its single biofilm form.

In summary, the interaction between bacterial species within the mixed biofilm conferred a survival advantage to *S. aureus*, enabling it to withstand the bactericidal effects of CHXD and resulting in an increased MBIC for both THY and CHXD. Previous studies have shown that the susceptibility of *S. aureus* to CHXD can differ in polymicrobial co-cultures[24–26]. It has been also reported that the presence of environmental selection pressure, such as antibiotics or the host immune system, stimulates a more synergistic relationship between different species and promotes biofilm formation. If *S. aureus* can withstand *P. aeruginosa* anti-staphylococcal activity

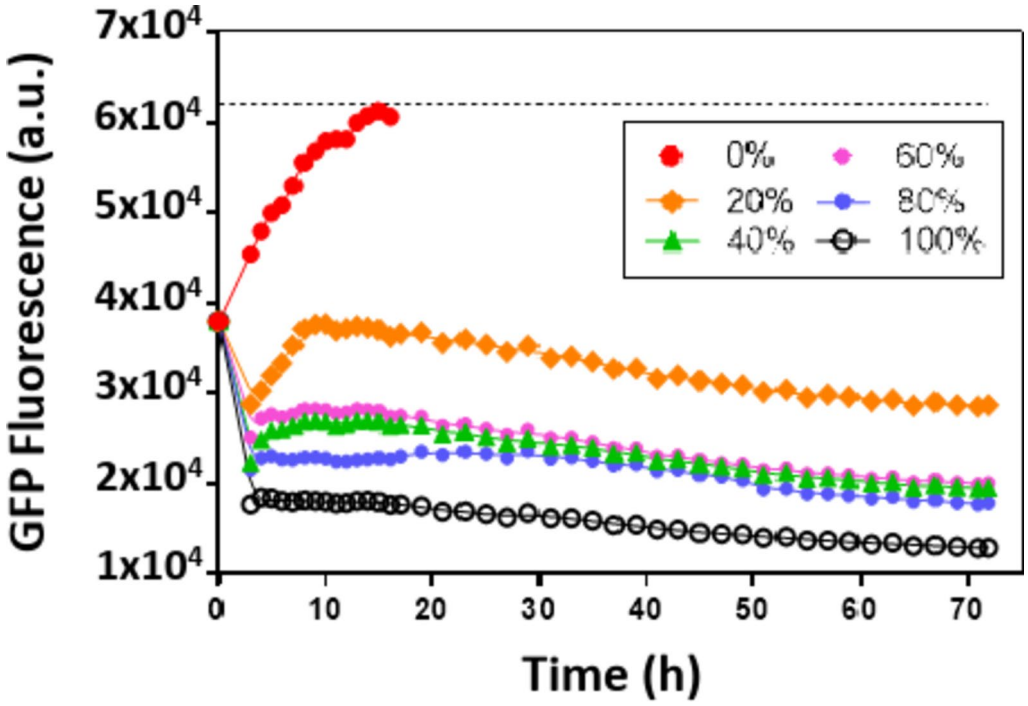


**Fig. 4**. *S. aureus* GFP fluorescence kinetics measured over 70 h after the addition of different percentages of conditioned medium (CM) obtained from an isolated culture of *P. aeruginosa* PAO1.

and successfully coexists within a multi-species biofilm, it gains an advantage from the protective antimicrobial barrier created by the matrix components of *P. aeruginosa*[27]. Based on the experimental work conducted, it can be concluded that in a clinical scenario where there were a mixed biofilm involving at least these two species, it would be better not only to disperse the biofilm extracellular matrix formed but also to focus on selectively eliminating *P. aeruginosa* before addressing *S. aureus*. Infected wounds are clinically treated with antiseptics but in those cases that the infection might be extended to the bone (i.e., osteomyelitis) systemic and topical antibiotics might be used. When having a polymicrobial biofilm the selection of a wide spectrum antibiotic is key to assure that both pathogenic species would be eliminated from the wound bed.

## Confocal microscopy studies

To further investigate the formation, interactions, and response to treatments within the established biofilm models, fluorescent bacterial strains (*S. aureus* GFP and *P. aeruginosa* BMP) were selected for their observation under confocal laser scanning microscopy (CLSM). Single biofilms of *S. aureus* and mixed biofilms of *S. aureus* with *P. aeruginosa* were analyzed, with the latter biofilms being developed according to the strategy depicted in Fig. 2a. As observed in Fig. 6a and b, single *S. aureus* biofilms were established by day 2. By day 4, mixed biofilms were established with *P. aeruginosa* interpenetrating the existing *S. aureus* network. These mixed biofilms appeared to be thicker compared to their single *S. aureus* counterparts under the same conditions.

On day 5, single *S. aureus* biofilms exhibited a slight increase in their thicknesses compared to day 4 and remained discernible, primarily owing to the presence of GFP fluorescence. In contrast, within the mixed biofilms, *S. aureus* GFP cells were no longer distinguishable due to the suppressive effects on the GFP fluorescence caused by *P. aeruginosa*.

To reveal that *S. aureus* was no more visible on the mixed biofilm at day 5 due to reduction of GFP expression, another set of untreated mixed biofilms was grown under the same conditions as those depicted in Fig. 2a, but with the addition of Syto9 after PI (propidium iodide). Syto9 is a green fluorescent nucleic acid dye capable of penetrating cell membranes, enabling the staining of all bacterial cells both live and dead in the biofilm non-specifically, revealing *S. aureus* that were no longer visible (Fig. 7).

According to the images, it was found that *P. aeruginosa* was aggregated within the biofilms, thus located significantly on the top part of the biofilm, compared to *S. aureus*. Comparing the orthogonal projections of the same sample after the addition of SYTO-9 the thickness of the stained bacteria layer increased, which is an indication of the presence of *S. aureus* embedded in the matrix but preferentially located on top of the *P. aeruginosa*. This observation could confirm the reported non-random distribution pattern of these bacteria within chronic wounds. Typically, *S. aureus* tends to colonize the superficial layers of the wound, while *P. aeruginosa* is found in the deeper regions of the wound bed. This spatial differentiation in colonization has also been reported in previous studies[28,29]. Some studies propose that *S. aureus* and *P. aeruginosa* interact early during infection to maximize their chances of colonization and enhance virulence, as this initial collaboration supports their establishment. However, once colonization is secured, both virulence and cooperation diminish, leading the bacteria to segregate into distinct niches[17].

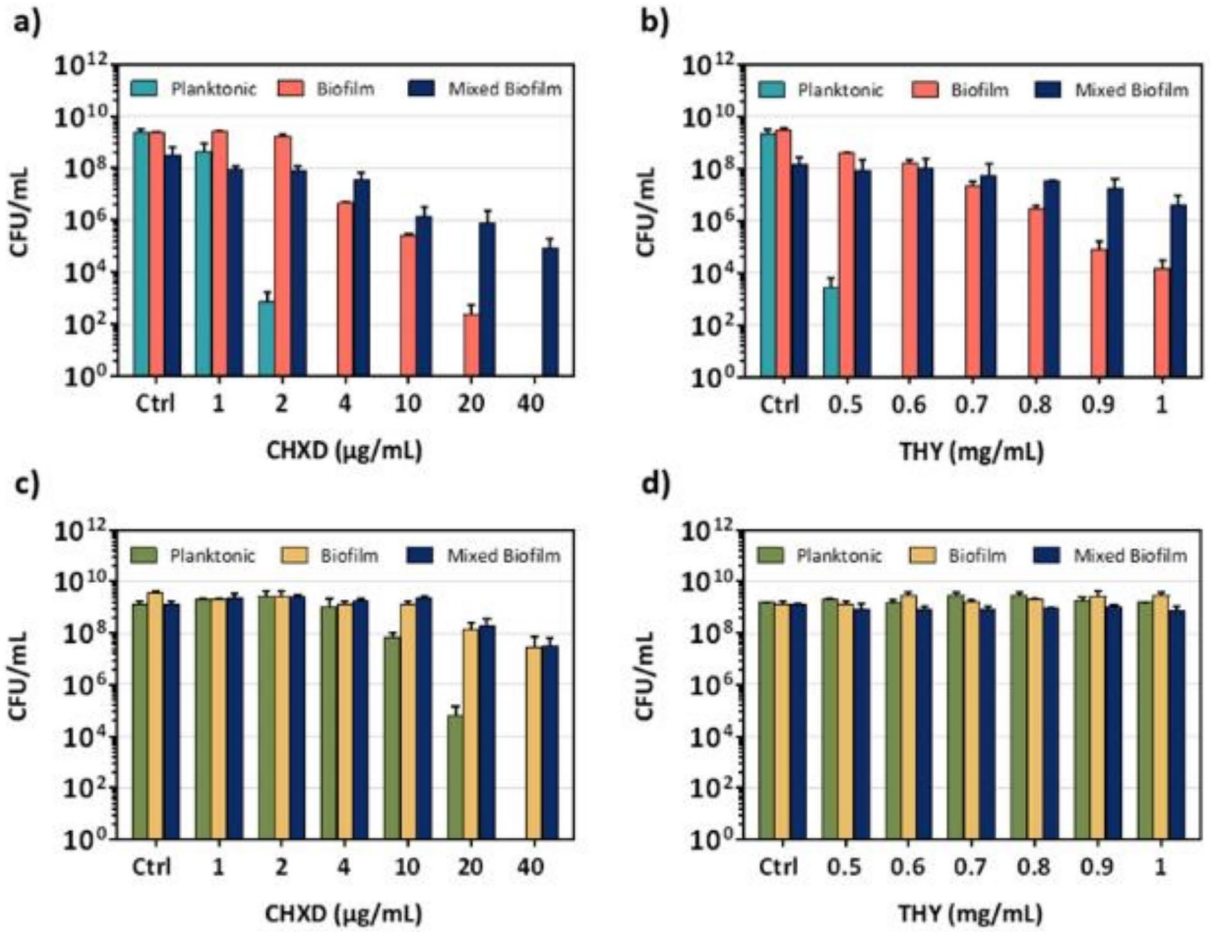


**Fig. 5**. Bactericidal activity of CHXD **(a)** and THY **(b)** against *S. aureus* Xen36 in planktonic, single-species biofilm and mixed biofilm formed with *P. aeruginosa* PAO1 after 24 h. Bactericidal activity of CHXD (**c**) and THY (**d**) against *P. aeruginosa* Lux in planktonic, single-species biofilm, and mixed biofilm formed with *S. aureus* 29213 after 24 h.

| | ***S. aureus* Xen36** | | | | ***P. aeruginosa* LUX** | | | |
|---|---|---|---|---|---|---|---|---|
| | **CHXD** | | **THY** | | **CHXD** | | **THY** | |
| | **MBIC (mg/L)** | **MBEC (mg/L)** | **MBIC (mg/L)** | **MBEC (mg/L)** | **MBIC (mg/L)** | **MBEC (mg/L)** | **MBIC (mg/L)** | **MBEC (mg/L)** |
| Planktonic | 2 | 4 | 500 | 600 | 20 | 40 | - | - |
| Biofilm | 4 | 40 | 700 | - | 40 | - | - | - |
| Mixed Biofilm | 10 | - | 1000 | - | 40 | - | - | - |

**Table 1**. MBIC and MBEC obtained for CHXD and THY against *S. Aureus* Xen36 and *P. aeruginosa* Lux in planktonic, single biofilm, and mixed biofilm states. (-) stands for non-reached at the maximum concentration tested.

Recent studies indicate that traditional swab culturing techniques may underestimate the presence of *P. aeruginosa* in wound infections[30]. Additionally, it aligns with the hypothesis that *P. aeruginosa* bacteria residing in the deeper regions of chronic wounds may play a pivotal role in maintaining wounds in a state characterized by inflammatory processes.

This work underlines the relevance of having polymicrobial populations and their microenvironment in the search of novel and effective treatments. It also highlights that the results obtained when evaluating an antimicrobial on planktonic bacteria or on single biofilms might be underestimated and the use of polymicrobial biofilms is recommended to mimic physiological settings.

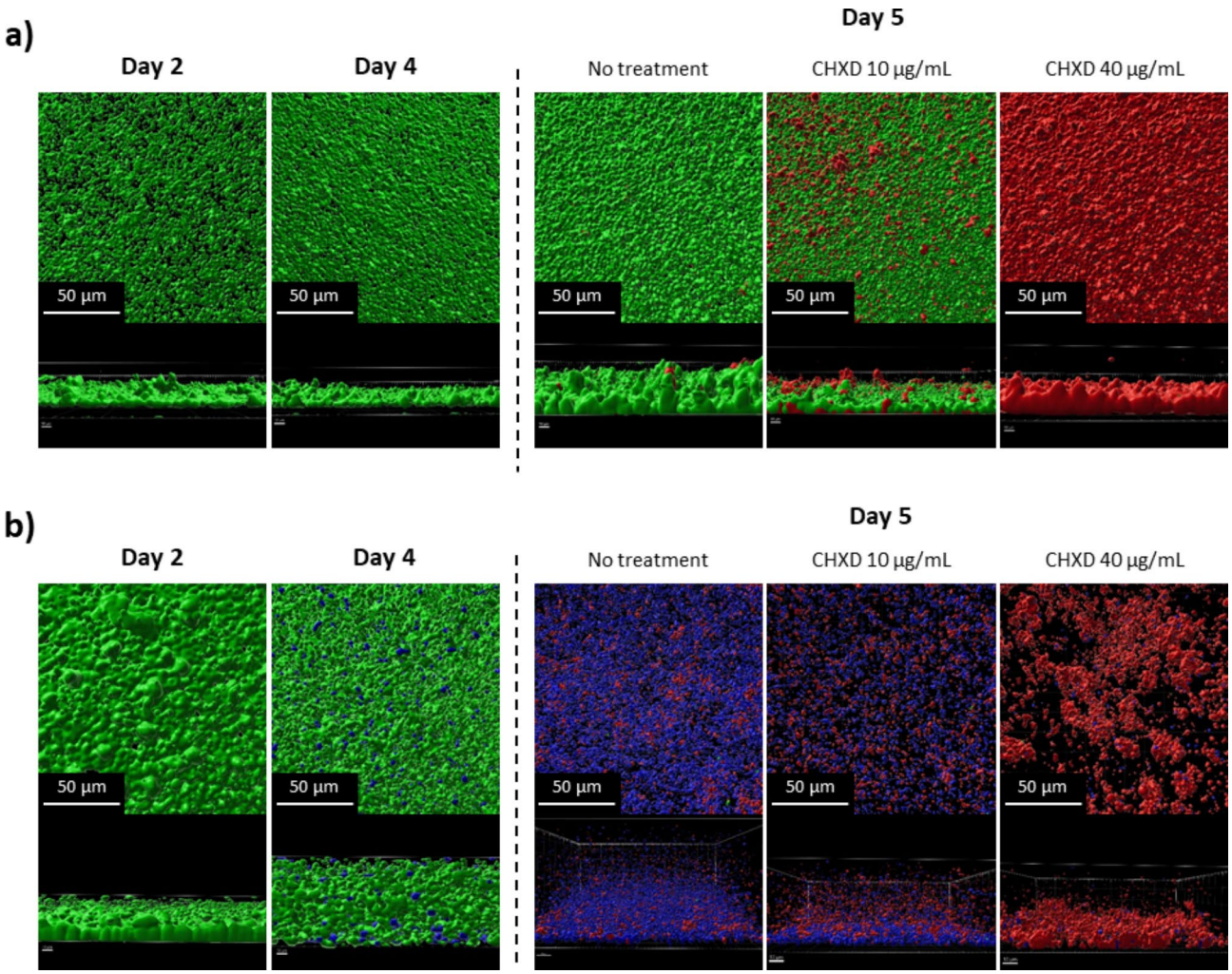


**Fig. 6**. CLSM biofilm visualization: **(a)** Single *S. aureus* GFP biofilm and **(b)** Mixed *S. aureus* GFP / *P. aeruginosa* BFP biofilm. Biofilms were visualized on days 2, 4 and 5 after treatment with 10 mg/L or 40 mg/L of CHXD. *S. aureus* GFP is emitting in green, *P. aeruginosa* BFP in blue, and dead bacteria in red, regardless of the species. Images were constructed from the z-stack acquisition.

## Materials and methods

### Materials

Thymol (THY, > 98.5%), chlorhexidine (CHXD, ≥ 99.5%), Mueller-Hinton broth (MHB), Mueller-Hinton agar (MHA) and sodium chloride (NaCl, > 99%) were purchased from Sigma-Aldrich (France). Three different *S. aureus* strains were used: *S. aureus* ATCC 29213; the bioluminescent *S. aureus* strain ATCC 49525 Xen36 (Perkin-Elmer, US); and a *S. aureus* strain expressing GFP (*S. aureus-GFP*; obtained using a pCN47 plasmid carrying a Phyper constitutive promoter as reporter of the GFP) originally from the laboratory of Dr. Iñigo Lasa and kindly donated by Dr. Cristina Prat (Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Spain). The *P. aeruginosa* strains used were: *P. aeruginosa* PA01 ATCC 27853; a bioluminescent *P. aeruginosa* PA01 strain obtained by the chromosomal integration of the LuxCDABE operon (*P. aeruginosa* Lux), supplied by Prof. Patrick Plesiat (Centre National de Référence de la résistance aux antibiotiques, Centre Hospitalier Universitaire de Besançon, France); and a fluorescent *P. aeruginosa* PA01 (ATCC 27853) strain transfected with mCTXtagBFP2, which expresses BFP (*P. aeruginosa-BFP*, blue fluorescence).

### Development of polymicrobial co-cultures

To obtain fresh liquid cultures of bacteria, isolated colonies of the tested strains were dispersed in 10 mL of MHB and incubated for 24 h under shaking at 37 °C. To produce a polymicrobial biofilm including the two bacterial species, two distinct microbiological strategies were used.

In the first strategy, a liquid culture of planktonic *S. aureus* (Xen36 or ATCC 29213) was diluted with fresh MHB to obtain ~ $10^7$ colony forming units (CFU)/mL ($OD_{600} \approx 0.006$). Then, 100 µL of the bacterial suspension were added to the wells of white, flat-bottom 96-well microplates. The luminescence of wells containing *S. aureus* Xen36 was recorded over time using a microplate reader (Infinite M200 Pro, Tecan®, Männedorf, Switzerland) until a value of around 1,000 relative light units (RLU) was reached, which allowed an increase or decrease

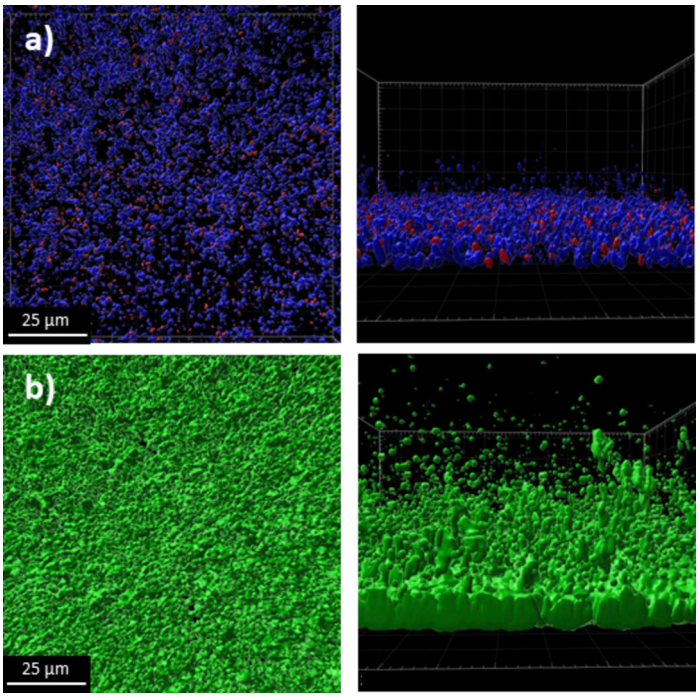


**Fig. 7**. CLSM images of mixed biofilms observed on day 5: **(a)** Before the addition of SYTO-9, where *P. aeruginosa* is stained in blue, dead bacteria in red, and the signal of *S. aureus* (green) is not appreciable. **(b)** The same biofilm after the addition of SYTO-9, which labels all bacteria both live and dead in green, including *S. aureus*. Images below were constructed from the z-stack acquisition.

in RLU to be detected later with good sensitivity. Next, 100 µL of bacterial suspension containing increasing concentrations ($10^1$ to $10^8$ CFU/mL) of planktonic *P. aeruginosa* Lux or *P. aeruginosa* PAO1 were added to the wells. To ensure that the bioluminescent signal was originated solely from only one of the two species, *P. aeruginosa* Lux was added to non-luminescent *S. aureus* ATCC 29213, while *P. aeruginosa* PAO1 was added to luminescent *S. aureus* Xen36. A visual representation of the experimental design is shown in Fig. 8a and b. After the addition of *P. aeruginosa* to *S. aureus*, the plates were sealed with a clear gas-permeable hydrophobic membrane (4titude Ltd, Surrey, UK) to prevent evaporation, and luminescence was recorded every 30 min for 48 h at 37 °C. Finally, the wells were carefully scraped and washed with 1 mL 0.9% (w/v) NaCl to harvest bacteria, which were serially diluted in 0.9% (w/v) NaCl and spread on two different agar media to obtain accurate bacterial counts of both species. MHA supplemented with colistin (8 mg/L) was used for the selective growth of *S. aureus*, and normal MHA for the growth of both species.

In the second approach, 100 µL of *S. aureus* suspensions (Xen36 or ATCC 29213) at $10^7$ CFU/mL were added to the wells of a white, flat-bottom, high-binding 96-well microplates. Plates were sealed with a hydrophobic gas-permeable membrane and incubated for 48 h at 37 °C under shaking. After biofilm formation, supernatants were removed, and the wells were washed 3-times with 0.9% (w/v) NaCl. Next, 100 µL of fresh MHB were added, and the bioluminescence was recorded until an intensity of around 1,000 RLU was achieved. Then, suspensions with increasing concentrations ($10^1$ to $10^8$ CFU/mL) of *P. aeruginosa* (Lux or PAO1) were added to the wells, as depicted in Fig. 8c and d. The microplates were then sealed with a clear gas-permeable hydrophobic membrane, and the luminescence was recorded every 30 min for 48 h at 37 °C. Finally, to collect the bacterial biofilms, the wells were carefully scraped, sonicated, and washed with 1 mL 0.9% (w/v) NaCl to harvest the bacteria, which were then serially diluted and seeded to count colonies.

## Study of the interaction between *S. aureus* and *P. aeruginosa* in mixed biofilm

To further investigate the interaction between *S. aureus* and *P. aeruginosa*, a Transwell® system having a 0.4 µm pore size polycarbonate membrane (Corning, France) was used to spatially separate both species. In brief, single biofilms of bioluminescent *S. aureus* Xen36 and mixed biofilms composed of *S. aureus* Xen36 and *P. aeruginosa* PAO1 were established at the bottom of 24-well plates using the strategy described in Fig. 8c. To form the mixed biofilms, 1 mL of *P. aeruginosa* PAO1 suspension at $10^4$ CFU/mL was inoculated onto 48 h old *S. aureus* Xen36 biofilms. On the other hand, the same mixed biofilms (*S. aureus* Xen36 and *P. aeruginosa* PAO1) or single PAO1 biofilms were formed onto Transwell® inserts as described above. Biofilms were then washed with 0.9% NaCl and fresh MHB was added (0.6 mL in the wells and 0.1 mL in the inserts). The inserts were placed in the wells and bioluminescence emitted by *S. aureus* Xen36 at the bottom of the wells was recorded at different times over 48 h using a microplate reader. At the end of the experiments, biofilms were disrupted via sonication, serially diluted, and plated on MHA to quantify *S. aureus* CFUs present in the wells.

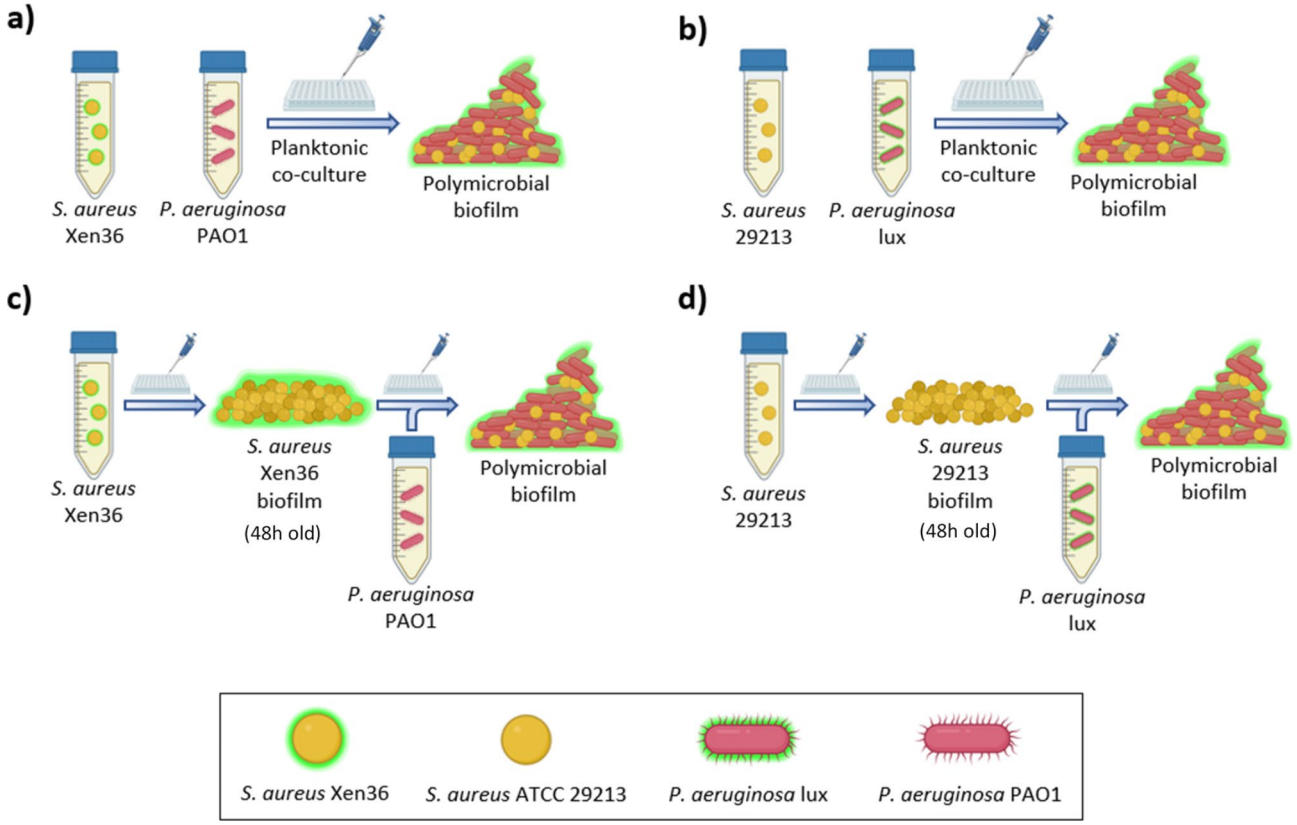


**Fig. 8**. Schematic overview of the strategies used to obtain polymicrobial biofilms: **(a)** and **(b)** schemes represent the first methodology, where the co-culture was established by adding the bacterial species in their planktonic state. **(c)** and **(d)** schemes depict the second methodology used, involving the addition of *P. aeruginosa* in the planktonic state to a pre-formed 48 h old *S. aureus* biofilm.

To determine whether *P. aeruginosa* was capable of secreting molecules interfering with the metabolic activity of *S. aureus* without being in direct contact or in proximity to it, a medium in which *P. aeruginosa* PAO1 had been cultured alone was collected and added to *S. aureus* biofilms. For this study, a mutant strain of *S. aureus* that expresses the green fluorescent protein (*S. aureus* GFP) was used. To obtain this conditioned medium (CM), 1.5 mL of *P. aeruginosa* PA01 at $10^4$ CFU/mL were seeded on top of 6-well Transwell® inserts placed in wells containing 2.6 mL of MHB and incubated in a water-saturated incubator at 37 °C. Three days later, the medium under the insert was collected, centrifuged twice at 13,000 g for 5 min and filtered through a 0.22 µm filter to remove any potential PAO1 bacteria present. This CM was then serially diluted and deposited on 48 h old *S. aureus* GFP biofilms grown in 96-well plates as explained above. *S. aureus* GFP fluorescence was then measured at 488 nm after excitation at 520 nm every hour for 72 h at 37 °C using a microplate reader.

## In vitro antimicrobial activity tests

Antimicrobial activity of THY and CHXD was tested against *S. aureus* Xen36 and *P. aeruginosa* Lux in planktonic state as well as when forming single and mixed biofilms.

To run tests against planktonic cultures, fresh bacterial cultures were adjusted to $10^7$ CFU/mL and 100 µL of the suspensions were added to the wells of white 96-well microplates. The bioluminescence of the wells was recorded over time (2–3 h) until reaching 1,000 RLU for *S. aureus* Xen36 and 10,000 RLU for *P. aeruginosa* Lux, which is more luminescent than *S. aureus* Xen36. Then, MHB solutions with increasing concentrations of CHXD (0.4 to 40 mg/L) and THY (400 to 1,000 mg/L) were added to reach a final volume of 200 µL. Next, the plates were sealed with an optical clear membrane and the luminescence was recorded every 30 min for 24 h at 37 °C. Finally, the cultures in the wells were seeded on MHA plates after serial dilution for CFU counting.

Regarding the experiments on single biofilm-forming bacteria, 100 µL of bacterial suspensions at $10^7$ CFU/mL were added to the wells of high-binding 96-well white microplates, which were then sealed with a gas-permeable membrane and incubated for 48 h at 37 °C with agitation. After incubation, MHB was removed, and biofilms were washed with 0.9% (w/v) NaCl before adding 100 µL of fresh MHB. The bioluminescence of the wells was then adjusted to the same values as the ones used for planktonic cultures (~ 1,000 RLU for *S. aureus* Xen 36, ~ 10,000 RLU for *P. aeruginosa* Lux). CHXD and THY treatments were also added to obtain the same concentrations as the ones used for evaluating the antimicrobial activity tests on planktonic bacteria (0.4 to 40 mg/L for CHXD and 400 to 1,000 mg/L for THY). Finally, the wells were thoroughly scraped and rinsed before serial dilution of the samples and plating them to quantify CFUs.

Mixed biofilm-forming bacteria assays were performed using suspensions containing $10^4$ CFU/mL of planktonic *P. aeruginosa* PAO1 and planktonic *P. aeruginosa* Lux, which were added to pre-formed 48 h old biofilms of *S. aureus* Xen36 and *S. aureus* ATCC 29213, as described in the previous section. These co-cultures were incubated again for 48 h to form the mixed biofilms before being washed with 0.9% (w/v) NaCl and before the addition of CHXD and THY treatments, as described previously for the individual biofilm activity tests. Lastly, mixed biofilms were dispersed, serially diluted, and plated on selective (colistin-supplemented MHA) and non-selective (regular MHA) agar plates to quantify CFUs.

### Confocal laser scanning microscopy studies

Single and mixed biofilms composed of the fluorescent *S. aureus* GFP and *P. aeruginosa*-BFP (Blue Fluorescent Protein) were grown on 8-well µ-slide chambers (Ibidi GmbH, Germany) enabling their spatial organization to be assessed by confocal laser scanning microscopy (CLSM). The strategy used for biofilms formation was identical to that described Fig. 8c, but fluorescent strains were used instead of the luminescent one. Briefly, 300 µL of *S. aureus*-GFP at $10^7$ CFU/mL were added to the chamber wells and incubated under shaking for 48 h at 37 °C in a water-saturated atmosphere for *S. aureus* biofilm formation. *S. aureus* biofilms were then washed and supplemented with MHB or $10^4$ CFU/mL of *P. aeruginosa* BFP suspension and incubated again for 48 h under the same conditions. Then, biofilms were washed and supplemented with different treatments (MHB, or MBH containing either CHXD 10 mg/L or 40 mg/L) and re-incubated for 24 h. The wells were then washed 3 times and filled with 300 µL of 0.9% NaCl containing propidium iodide (PI) at 20 µM. Some mixed biofilms were also labelled with 5 µM SYTO-9, a fluorescent probe that labels both live and dead bacteria in green.

At different times (2, 4 and 5 days), biofilms were visualized under an Olympus FluoView FV-3000 with an IX83 CLSM (x100 zoom). For each biofilm, samples were sequentially excited to study the fluorescence of GFP or SYTO-9 (excitation 488 nm - emission 500/540 nm), BFP (excitation 405 nm - emission 430/470 nm), and PI (excitation 561 nm - emission 570/670 nm). Twenty to thirty stacks of horizontal plane images (1024 × 1024 pixels corresponding to 127 × 127 µm) with a z-step of 0.5 µm were acquired for each condition. Three-dimensional biofilm projections were constructed using the Easy 3D function of the IMARIS software (Bitplane). When creating the surfaces, the same segmentation parameters (particle size, absolute intensity and absence of post-segmentation filters) were applied to all 3D constructs.

## Conclusion

The monitoring of co-cultures using bioluminescent, fluorescent and wild type bacterial strains, in order to develop a polymicrobial mixed biofilm, was successful. Notably, *P. aeruginosa* was able to secrete molecules that repress *S. aureus* metabolism and protein synthesis without requiring physical contact or the presence of both species in the same environment.

In mixed biofilms, the interaction between the two bacterial species provided *S. aureus* with a survival advantage, making it less susceptible to the bactericidal effects of CHXD and resulting in elevated MBIC values for both THY and CHXD. Lastly, the non-random spatial organization of the bacterial species observed within the mixed biofilm had similarity with previous reports on co-infected chronic wounds. This alignment underscores the importance of bacterial crosstalk in polymicrobial cultures and highlights the potential implications for understanding and managing polymicrobial wound infections to select the most appropriate antimicrobial regime.

## Data availability

The data will be available from the corresponding authors after reasonable request.

Received: 17 August 2024; Accepted: 11 November 2024
Published online: 13 November 2024

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## Acknowledgements

The authors acknowledge the Spanish Ministry of Science and Innovation (grant numbers PID2020-113987RB-I00 and PDC2021-121405-I00) for funding. G.L. acknowledges the support from the FPI program (PRE2018-085769, Spanish Ministry of Science, and Innovation). G.M. gratefully acknowledges the support from the Miguel Servet Program (MS19/00092; Instituto de Salud Carlos III). A portion of the schemes depicted in figures were created with the assistance of BioRender.com.

## Author contributions

Conceptualization: F.T.; Methodology: G.L., J.C. and J.B.; Formal analysis: G.L., F.T.; Investigation: G.M., M.A. and F.T.; Writing original draft: G.L. and G.M.; Writing, Review and Editing: J.C., J.B., M.A. and F.T.; Supervision: G.M., M.A. and F.T.; Funding acquisition: M.A.

## Declarations

## Competing interests

The authors declare no competing interests.

## Additional information

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