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NaCl-induced modulation of species distribution in a mixed *P. aeruginosa* / *S. aureus* /*B. cepacia* biofilm

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ABSTRACT

Pseudomonas aeruginosa, *Staphylococcus aureus*, and *Burkholderia cepacia* are notorious pathogens known for their ability to form resilient biofilms, particularly within the lung environment of cystic fibrosis (CF) patients. The heightened concentration of NaCl, prevalent in the airway liquid of CF patients' lungs, has been identified as a factor that promotes the growth of osmotolerant bacteria like *S. aureus* and dampens host antibacterial defenses, thereby fostering favorable conditions for infections.

In this study, we aimed to investigate how increased NaCl concentrations impact the development of multi-species biofilms *in vitro*, using both laboratory strains and clinical isolates of *P. aeruginosa*, *S. aureus*, and B. cepacia co-cultures. Employing a low-nutrient culture medium that fosters biofilm growth of the selected species, we quantified biofilm formation through a combination of adherent CFU counts, qPCR analysis, and confocal microscopy observations.

Our findings reaffirmed the challenges faced by *S. aureus* in establishing growth within 1:1 mixed biofilms with *P. aeruginosa* when cultivated in a minimal medium. Intriguingly, at an elevated NaCl concentration of 145 mM, a symbiotic relationship emerged between *S. aureus* and *P. aeruginosa*, enabling their co-existence. Notably, this hyperosmotic environment also exerted an influence on the interplay of these two bacteria with *B. cepacia*. We demonstrated that elevated NaCl concentrations play a pivotal role in orchestrating the distribution of these three species within the biofilm matrix.

Furthermore, our study unveiled the beneficial impact of NaCl on the biofilm growth of clinically relevant mucoid *P. aeruginosa* strains, as well as two strains of methicillin-sensitive and methicillin-resistant *S. aureus*. This underscores the crucial role of the microenvironment during the colonization and infection processes. The results suggest that hyperosmotic conditions could hold the key to unlocking a deeper understanding of the genesis and behavior of CF multi-species biofilms.

1. Introduction

Cystic fibrosis is the most common inherited genetic disease in Caucasian populations, and it is characterised by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1]. The dysfunction of CFTR protein leads to reduced chloride, bicarbonate, and water secretion from epithelial cells into the airway surface liquid (ASL) [2–4]. Subsequent water retention within the epithelium becomes accountable for the buildup of desiccated and thickened mucus within the airways. This, in turn, disrupts the process of mucociliary clearance [1,5]. Impaired mucus clearance in individuals with CF promotes bacterial colonization and triggers tissue inflammation [5]. In younger patients, lung infections primarily involve bacteria like *Staphylococcus aureus* or *Haemophilus influenzae*. However, as the disease advances, there's an increasing prevalence of infections caused by opportunistic Gram-negative pathogens, such as *Pseudomonas aeruginosa*, *Burkholderia cepacia*, or *Stenotrophomonas maltophilia* [6]. Among lung pathogens, *S. aureus* and *P. aeruginosa* stand out as the most prevalent, often found co-existing in CF patients. Unfortunately, these co-isolations are associated with worse outcomes compared to infections involving *P. aeruginosa* alone [7–10]. To date, the relationship between *P. aeruginosa* and *S. aureus* and the findings that came out from related

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studies remain controversial, as reviewed recently by Bow Yue Yung *et al.* [11].

Alongside *P. aeruginosa* and *S. aureus*, bacteria belonging to the *B. cepacia* complex are commonly detected in sputum analyses. Although it is responsible for only 5% of the total CF infections, *B. cepacia* is often associated with a fatal prognosis [12]. What adds to the concern is the observation that these pathogens have been co-isolated in a substantial proportion of cases, raising questions about potential interactions and their combined impact on health outcomes of CF patients [13,14].

P. aeruginosa, *S. aureus*, and *B. cepacia* exhibit a common proficiency in colonizing and thriving within the respiratory tract through the formation of biofilms [15–17]. Within this distinct lifestyle, bacteria organize into communities that frequently exhibit resistance to immune reactions and antibiotics. This resistance is achieved through a combination of inherent, acquired, and biofilm-related mechanisms, rendering them multi- and pan-resistant.

Over the past decade, numerous biofilm models have emerged to delve into the intricacies of the mechanisms underlying biofilm formation [18], the evaluation of antibiotics susceptibility [19–21], or in the search of anti-biofilm agents with novel mechanisms for the treatment of CF [22–24]. However, most of the studies were based on single-species *in vitro* models, most often *P. aeruginosa* biofilms. Given that the bacteria accountable for infections in CF patients have been observed to flourish as multi-species biofilms, the translation of anti-biofilm strategies from laboratory models to clinical settings could pose substantial challenges [25]. Consequently, recent reviews have underscored the significance of accounting for mixed biofilms, particularly when assessing antimicrobial susceptibility [25–27].

Beyond the influence of the microbiome, environmental factors have demonstrated their paramount significance in bacterial colonization within CF patients. For example, a connection has been established between the gradual lung colonization by *P. aeruginosa* and the worsening of the infection, alongside an elevation in chloride concentration within the airway surface liquid [3,28]. At the same time, *S. aureus* osmotolerance is well documented and high NaCl levels are known to stimulate the formation of *S. aureus* biofilms [29,30]. Furthermore, sodium chloride (NaCl) is thought to wield a significant impact on CF, as evidenced by the correlation between elevated NaCl concentrations found in the airway surface liquid (ASL) and the unfavorable progression of the infection [31]. Hence, comprehending the behavior and tolerance of pathogens in response to elevated NaCl levels becomes paramount for uncovering novel anti-biofilm strategies in CF. Despite the introduction of various synthetic CF media (SCFM) aimed at mimicking natural CF sputum composition, this particular parameter remains relatively underexplored [32–35]. Here we focused on assessing the effect of high NaCl concentrations on the growth of multi-species biofilms, considering both reference strains and clinical isolates of *P. aeruginosa*, *S. aureus*, and *B. cepacia*.

Drawing from prior research, biofilms were cultivated within a minimal culture medium, specifically a low-nutrient broth. Following the recommendation of Bow Yue Yung *et al.* [11], this approach aimed to replicate the naturally nutrient-restricted growth environment, promoting the prevalence of biofilm populations over planktonic bacteria [36,37].

2. Results

2.1. Study of PA-SA two-species biofilms

In this study, PA-SA biofilms were cultivated within the low-nutrient GBB medium. After 48 h, quantification of colony-forming units (CFUs) revealed that the population of adhered PA cells remained consistent (~7 Log CFU/mL) whether cultivated in mono- or dual-species setups at a 1:1 PA/SA ratio (Supplementary Figure 2 and Fig. 1A, respectively). Conversely, a noteworthy reduction of 3.7 Log CFUs in SA was observed at the 48-h mark between mono- and dual-species biofilms (Supplementary Figure 1 and Fig. 1A, respectively), resulting in a calculated competitive index (CI) of 0.42. This CI was derived by dividing the SA/PA output ratio by the input inoculum ratio. A CI greater than 1 signifies a higher abundance of SA in the dual-species biofilm [38]. Hence, in order to sustain the adherent SA population within the dual-species biofilm, various SA inoculum ratios were examined (Fig. 1B). Microplates containing GBB medium were subjected to an initial inoculation with a 10^2 CFU/mL suspension of PA. Meanwhile, SA suspensions were introduced at varying concentrations, ranging from 10^2 CFU/mL to 5.10^4 CFU/mL (equivalent to 10 to 5.10^3 CFU per well). Subsequent to 24 and 48 h of incubation, the adhered cell counts were enumerated.

Initial observations revealed that SA exhibited the capacity to sustain substantial levels of adherent cells after 24 h of co-incubation with PA, ranging from 5.6 to 6.2 Log CFU/ml. However, a notable decline was evident after 48 h, particularly noticeable at low SA inoculum levels, particularly with a 1:10 ratio (CI = 0.24). Interestingly, even at a 48-h mark, the population of viable SA adherent cells managed to remain robust, registering up to 6 Log CFU/mL (CI = 0.17), particularly when co-cultivated in a 1:500 PA/SA ratio (Fig. 1B).

To delve deeper into the behaviors of each population within the dual-species environment, and to draw comparisons with their respective single-species counterparts, we employed confocal microscopy to examine PA/SA 1:500 biofilms (Fig. 2A and B). Employing live/dead staining using Syto9 (green) and propidium iodide (PI, red), we observed a predominance of live cells, accompanied by a limited number of deceased cells. With the utilization of Syto9/hexidium iodide (HI) Gram staining, we discovered a fairly balanced distribution of bacteria, with Gram-negative (54.0% of fluorescence) and Gram-positive (46.0%) organisms encompassing the entire biofilm (Fig. 2B and Table S1).

2.2. The influence of osmotolerance in SA and PA on the formation of dual-species biofilms

Given the significance of ion balance in CF lung infections, our subsequent focus was on examining how elevated NaCl concentrations impact biofilm establishment and the behavior of the respective populations. Our initial inquiry delved into the characteristics of both single and dual-species biofilms involving PA and SA. For mixed biofilms, we employed a simultaneous 1:500 co-culture inoculation (at t0). The selection of the three NaCl concentrations aimed to closely approximate those recorded in CF sputum samples [33]. The GBB medium supplemented with NaCl exhibited a pH ranging from 6.9 to 7.1, which is in close proximity to that of other synthetic media [20,33,39] (Table 1). The adhered population was evaluated by CFU counting and by qPCR quantification (Fig. 3). Statistical analysis was performed between each sample and the control (*), and between CFU and genomic units (GU) data (°).

- One-species biofilms:

Quantifying CFUs in PA biofilms revealed a notable rise in the adherent population when exposed to 290 mM NaCl (+1.1 Log CFU/mL) in contrast to the control in GBB medium (Fig. 3A). This outcome was validated through qPCR quantification, yet the heightened levels of quantified biomass implied the presence of non-cultivable or deceased cells, as substantiated by confocal observations (PI staining, Fig. 2A). Regarding SA biofilms, the population of adherent cells exhibited a considerable increase at 72.5 mM NaCl (7.16 ± 0.48 Log CFU/mL) in contrast to the control (6.52 ± 0.22 Log CFU/mL), and this level remained consistent at both 145 and 290 mM (Fig. 3B). Contrasting the outcomes from the two quantification techniques did not unveil any notable impact of NaCl on cell viability, a finding that was corroborated by confocal microscopy (Fig. 2C).

- Dual-species biofilms cultivation:

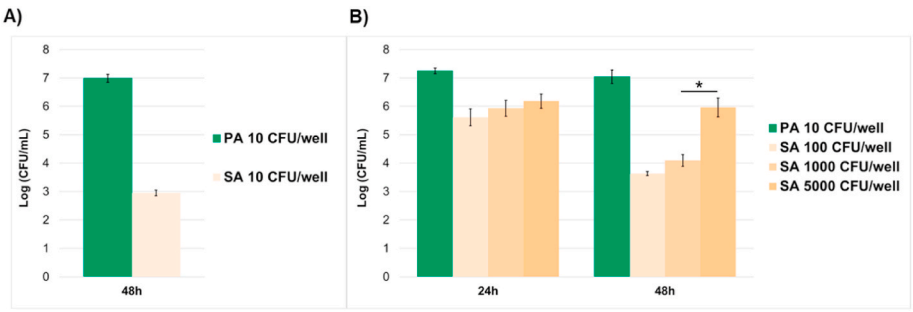

Fig. 1. Growth as two-species biofilms of PA and SA after 24 h and 48 h, in the GBB medium. Two-species biofilms where inoculated A) with a 1:1 bacterial ratio at 10 CFU/well; B) with different SA inoculum concentrations, varying from 10^2 CFU/well to 5.10^3 CFU/well (mean ± standard deviation; n = 2*3). *$p < 0.05$.

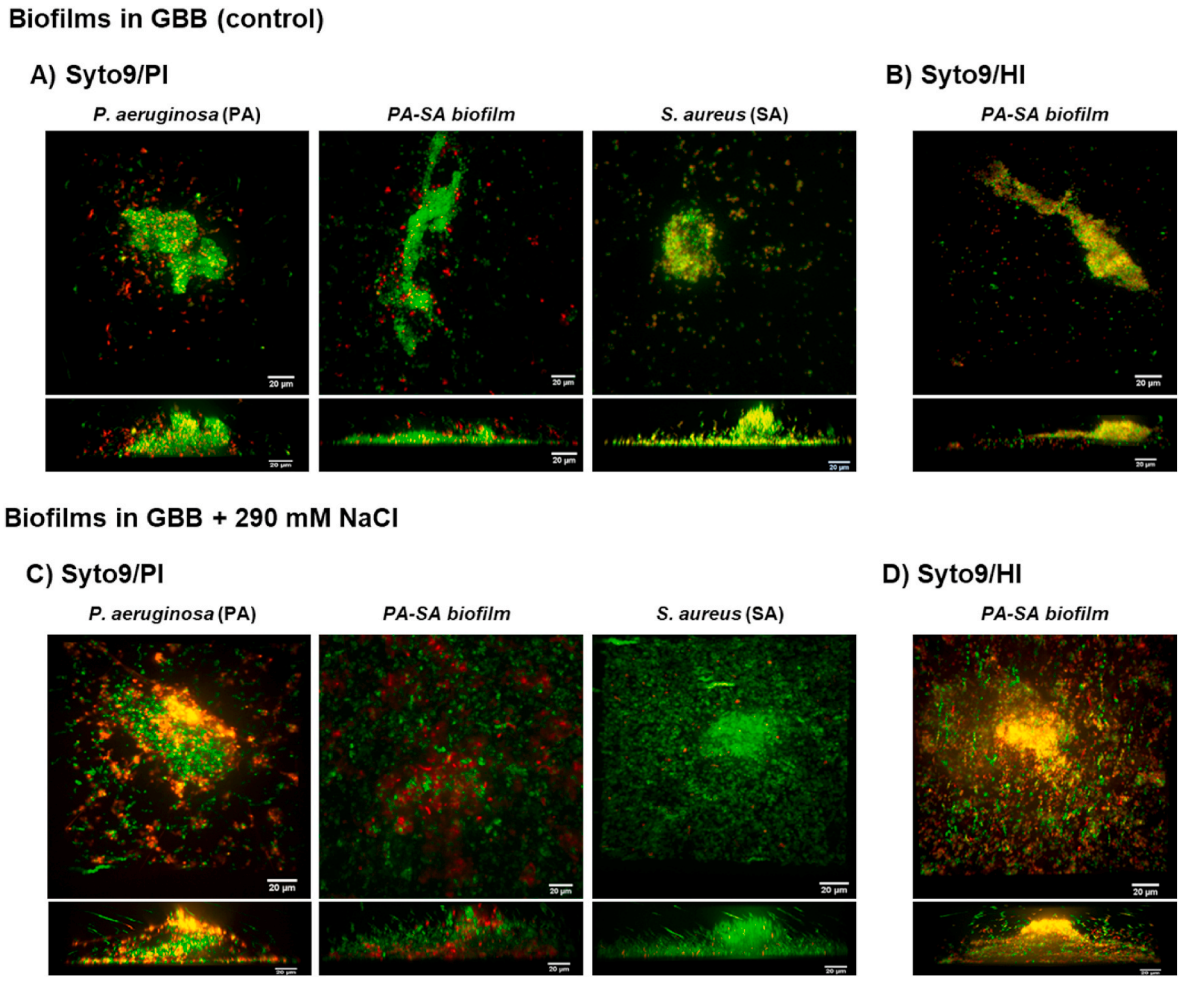

Fig. 2. Confocal Laser Scanning Microscopy (CLSM) images of PA, SA and PA/SA two-species biofilms, (inoculation ratio 1:500, 48 h) in GBB and GBB supplemented with 290 mM NaCl A) and C) Syto9/PI staining (Live/Dead), B) and D) Syto9/HI staining (Gram – (green)/Gram + (red)) of PA/SA biofilms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The cultivation of dual-species biofilms was carried out under the previously defined conditions, as mentioned above. This involved utilizing a PA:SA inoculum ratio of 1:500 at the outset (t0), while also varying NaCl concentrations. The outcomes for PA quantification (CFU counts) closely mirrored those observed in the context of one-species biofilms. This reaffirmed the dose-dependent influence of NaCl on the formation of PA biofilms (Fig. 3C).

In terms of SA, the control results corroborated the adverse impact of PA on the adhered SA population (as evidenced by CFU counts), manifesting as a ~2.1 Log reduction after 48 h of incubation. This reduction in the SA population was similarly detected through qPCR quantification, albeit to a lesser degree (~1.1 Log reduction).

Remarkably, the presence of 72.5 mM NaCl fostered the maintenance of the SA population. Notably, this led to a significant 3.27 Log CFU/mL increase in the SA population compared to the control. This positive effect of NaCl on SA persistence persisted at 145 and 290 mM NaCl concentrations. Analyzing dual-species biofilms through confocal microscopy (Syto9/HI staining) revealed a marked augmentation in the SA population when exposed to 290 mM NaCl. This increase was indicated by the elevation in HI fluorescence, shifting from 46.0% for the control

Table 1
Comparison of composition in ions, sugars, total free amino acids, and pH of CF sputa, two synthetic media from literature (SCFM2 and ASM), and the NaCl-supplemented GBB used in the present study.

[C] (mM)	CF sputum samples[a]	SCFM2[b]	ASM[c]	GBB	GBB + 72.5 mM NaCl (0.425% w/v)	GBB + 145 mM NaCl (0.85% w/v)	GBB + 290 mM NaCl (1.70% w/v)
Na^+	37.0–100.6	66.6	85.5	17.6	90.1	162.6	307.6
K^+	9.5–18.5	15.8	29.5	3.7	3.7	3.7	3.7
Cl^-	16.4–158.9	79.1	114.5	0.004	72.5	145.0	290.0
Glucose	1.3–4.5	3.2	1%	2.2			
Other sugars and carbon sources	Lactate 3.6–15.0	Lactate 9.0	Egg yolk emulsion	Lactose 0.073			
Total free amino acids	100.8 mM	18.48 mM	5 g/L (Becton, Dickinson)	0.47 mM			
Mucin		5 g/L	10 g/L				
DNA			4 g/L				
pH	6.0–6.9	6.8	7.0	7.2	7.1	7.0	6.9

[a] [33].
[b] [35].
[c] [20].

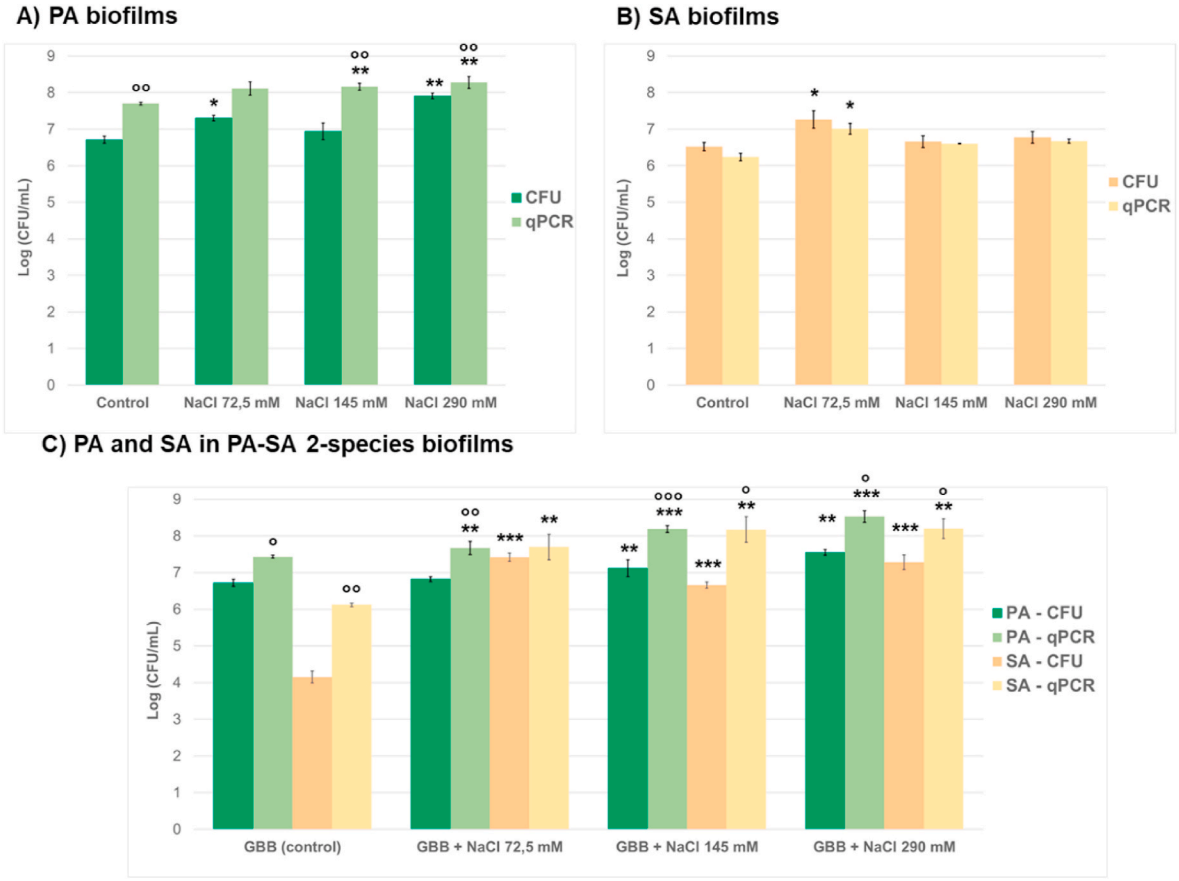

Fig. 3. Biofilm quantification by CFU and qPCR after 48 h of (A) PA and (B) SA in one-species biofilms and (C) in two-species biofilms in GBB (control) and NaCl-enriched GBB medium. Inoculum ratio 1:500) (mean±standard deviation; one-species: n = 2*2; two-species: n = 3*3). Statistical analysis was performed by comparing the results for each NaCl concentration with their respective controls, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Further statistical analysis comparing GU data and Log (CFU/mL) data from the same growth conditions are represented as follows: °$p < 0.05$; °°$p < 0.01$; °°°$p < 0.001$.

to 56.1% (Fig. 2D and Table S1).

2.3. NaCl restores SA population in a 1:1 PA/SA biofilm

Given the positive impact of NaCl on the adherent SA population within the 1:500 dual-species biofilms, we extended our investigation to assess the effect of 145 mM NaCl in a 1:1 PA/SA co-culture. Under these conditions, the adhered SA population exhibited a notable increase (+2.83 Log CFU/ml) compared to the control in GBB (Fig. 4). This increase was paralleled by an elevation in the competitive index (CI) from 0.41 to 0.77. These findings underscore that NaCl plays a role in empowering SA to outcompete PA, thereby enabling the maintenance of a viable adherent population over time.

For a more comprehensive understanding of SA's settlement within biofilms, we undertook a sequential bacterial inoculation strategy involving PA on SA biofilms. This assay aimed to mimic *in vivo* scenarios by introducing PA (at a concentration of 10^2 CFU/mL at t0) onto a pre-established SA biofilm (initial inoculation at 5.10^4 CFU/mL) that had

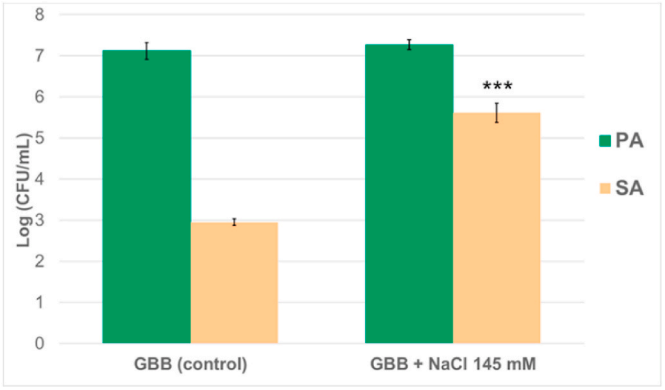

Fig. 4. Quantification of two-species PA-SA biofilms after 48 h growth in GBB or GBB supplemented with 145 mM NaCl. (Inoculation ratio PA/SA 1:1) (mean ± standard deviation, n = 2*2). ***p < 0.001.

been allowed to develop over 48 h. The objective was to ascertain whether PA would eventually outcompete SA over time. At the conclusion of the experiment (48 h after PA inoculation onto the pre-established SA biofilm), the adherent cells (CFU) of both PA and SA were quantified (Fig. 5).

The results unveiled that PA sustained growth patterns similar to those observed in one-species biofilms. However, the number of SA adherent cells experienced a notable decline after 48 h (−3.2 Log CFU/mL). This observation affirmed that PA progressively exerts an adverse influence on the adherent SA population during biofilm growth.

In the presence of 145 mM NaCl, the quantified population of adhered SA cells remained stable after 48 h. Importantly, this population showed no significant difference when compared to the SA one-species control (Fig. 5C), thereby confirming that NaCl fosters the growth of SA.

Collectively, these outcomes collectively indicate that an elevated NaCl concentration contributes to enhanced SA settlement and sustenance within mixed communities alongside PA. This is true whether the inoculation of the two species occurs simultaneously or sequentially.

2.4. Two-species biofilms of PA and SA clinical strains

To validate the potential influence of NaCl in the colonization of CF patients' lungs by strains of clinical relevance, we examined three isolates obtained from CF patients (Table 2. These isolates comprised a mucoid *P. aeruginosa* (mPA), a methicillin-sensitive *S. aureus* (MSSA), and a methicillin-resistant *S. aureus* (MRSA). Notably, the mucoid *P. aeruginosa* and the MSSA strains were both sourced from the same patient.

- One-species biofilms (Fig. 6A):

The quantification of adhered mPA cells after 48 h in the GBB medium yielded a lower count of cultivable cells (5.46 ± 0.77 Log CFU/ml) compared to the PA laboratory strain (6.71 ± 0.19 Log CFU/ml). Interestingly, in contrast, a notable increase in biofilm biomass was evident in the presence of 145 mM NaCl (~+1.4 Log CFU/ml), in

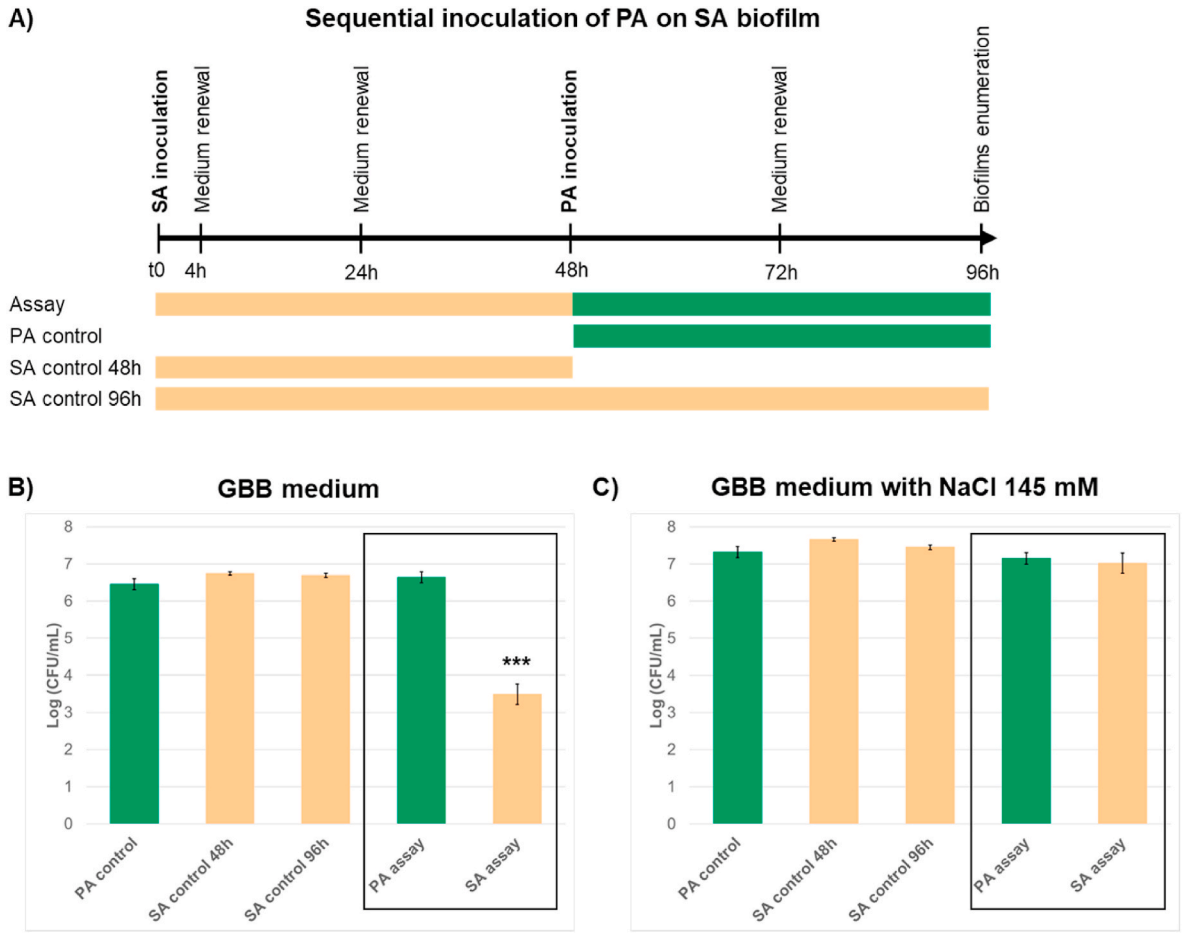

Fig. 5. Inoculation of 48 h-old SA biofilm by PA in GBB medium A) Experimental diagram of sequential SA and PA inoculation (PA/SA 1:500), and CFU results **B)** in GBB medium and **C)** in GBB medium with 145 mM NaCl (mean ± standard deviation; n = 2*3). Statistical analysis was performed between mixed biofilm data and the corresponding control at 48 h, ***p < 0.001.

Table 2
Strain abbreviations used in this study.

Strain name	Abbreviation	Patient's characteristics
P. aeruginosa PAO1 (CIP 104116)	PA	
MR *S. aureus* (ATCC ® 33591™)	SA	
B. cepacia (ATCC® 25416™)	BC	
mucoid *P. aeruginosa*, clinical strain (Toulouse Hospital)	mPA	patient n° 1, age 27 colonization by SA and PA for more than a year
MSSA, clinical strain (Toulouse Hospital)	MSSA	3 strains detected: 1 MSSA; 2 PA (one mucoid, one non-mucoid); no BC detected
MRSA, clinical strain (Toulouse Hospital)	MRSA	patient n° 2, age 23 colonization for more than a year by SA; only 2 strains detected: 1 MRSA; 1 MSSA no BC and no PA detected

comparison to the PA laboratory strain (Fig. 3A and Fig. 6A).

Regarding the MSSA and MRSA strains, their growth was quite comparable (6.20 ± 0.33 and 7.03 ± 0.11 Log CFU/mL, respectively) to that of the SA laboratory strain (6.52 ± 0.22 Log CFU/mL). Similar to what was observed for the SA reference strain, a concentration of 145 mM NaCl did not produce a significant effect on the MSSA and MRSA monospecies biofilms.

- Dual-Species mPA/MSSA and mPA/MRSA Biofilm Formation:

Dual-species biofilms of mPA/MSSA and mPA/MRSA were established through co-inoculation (1:1) in the GBB medium, with or without the addition of 145 mM NaCl (Fig. 6B–C).

In the absence of NaCl, the presence of mPA did not appear to impact the growth of the two clinical SA strains. The adhered cell levels remained similar to those observed in one-species biofilms, suggesting that the tested clinical mPA strain did not outcompete either of the SA strains. After 48 h of co-incubation, the competitive index (CI) for the mPA/MSSA and mPA/MRSA biofilms were 1.04 and 1.23, respectively. This indicates a slight dominance of the MRSA strain over the mPA strain in the GBB medium.

With the introduction of 145 mM NaCl, a noteworthy rise in the mPA cell population was observed in both mixed biofilms, reaffirming the positive influence of NaCl on the formation of PA biofilms. Importantly, the mucoid phenotype of mPA remained intact in the presence of NaCl, both in single and dual-species biofilms. Additionally, a significant increase in the MSSA adhered population (~0.6 Log CFU/mL) was noted (Fig. 6B), indicating a favorable impact of NaCl on both populations originating from the same patient. In contrast, NaCl did not significantly affect the growth of the MRSA strain (Fig. 6C).

Remarkably, in these conditions, the populations were well-balanced within both biofilms. The calculated CIs were 0.97 and 0.94 for the mPA/MSSA and mPA/MRSA biofilms, respectively, highlighting the absence of a competitive advantage for either species.

2.5. Multi-species experiments and B. Cepacia inclusion

Incorporating *B. cepacia* into the experiments added another dimension to our study, considering it is often identified in CF-associated lung infections [86,87]. To begin, we examined 1:1 co-cultures in GBB, investigating interactions between BC and PA and SA, respectively (Supplementary Fig. 4). In the case of PA/BC biofilms, both PA and BC adhered populations remained stable at 7.19 ± 0.05 and 7.57 ± 0.15 Log CFU/mL, respectively. This finding underscores that these two Gram-negative species thrive together within biofilms (Supplementary Fig. 4B). In the SA/BC 48-h biofilms, the quantification of

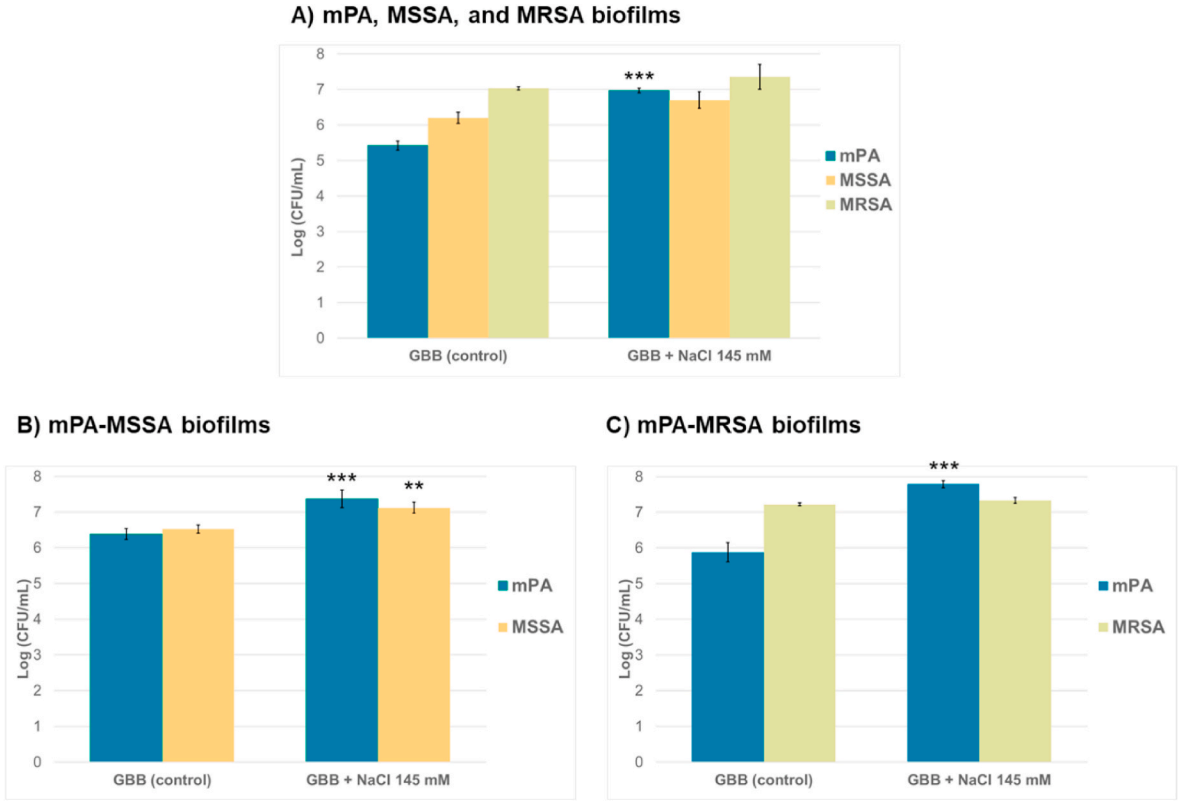

Fig. 6. Quantification of clinical strains biofilms after 48 h in GBB and GBB supplemented with 145 mM NaCl A) mPA, MSSA and MRSA one-species biofilms, B) mPA-MSSA biofilms (inoculum ration 1:1), and C) mPA-MRSA biofilms (inoculum ration 1:1) (mean ± standard deviation; n = 2*3). **p < 0.01; ***p < 0.001.

adhered cells exhibited a significant reduction in SA cells (a 2.2 Log reduction), while the BC population remained unchanged (Supplementary Fig. 4C). Interestingly, this behavior mirrored what was observed in PA/SA biofilms in GBB.

Advancing our exploration, we ventured into three-species biofilms encompassing PA, SA, and BC in GBB medium for 48 h, using a PA/SA/BC 1:500:1 inoculation ratio (Fig. 7). The outcomes revealed that SA was the least abundant species, registering 3.32 ± 0.90 Log CFU/mL, while the other two species exceeded 6 Log CFU/mL after 48 h.

In the presence of 145 mM NaCl, a substantial shift in species distribution was noted. While the PA population remained steady, the adhered SA population surged by 4.05 Log and took on a dominant role. Conversely, BC emerged as the least abundant species, experiencing a decline of 0.75 Log in adhered cells. Furthermore, observations through confocal microscopy demonstrated an increase in Syto9 fluorescence from 64.5% to 74.0%, indicating a growth of the Gram-positive cell population in the presence of 290 mM NaCl (Fig. 8D and Table S1) when compared to the control (Fig. 8B and Table S1). These findings solidify that NaCl exerts a positive effect on the capacity of SA to thrive within mixed biofilms.

Since NaCl had no significant impact on one-species BC biofilms (Supplementary Fig. 4A), these results suggest that the decrease in the BC sessile population in the presence of NaCl primarily arises from shifts in species interactions within the biofilm, rather than a direct inhibitory effect of NaCl on BC growth.

3. Discussion

Cystic fibrosis-related infections predominantly stem from the colonization of the lungs by various biofilm-producing pathogens, notably *Pseudomonas aeruginosa* (PA), *Staphylococcus aureus* (SA), and *Burkholderia cepacia* (BC). As patients age and the disease advances, there's a gradual transition from SA to PA, although SA's presence is not entirely supplanted and often persists alongside PA in around 65% of cases [9]. Simultaneously, cystic fibrosis-associated infections involving *B. cepacia* are frequently linked to a grave prognosis [40,41]. What adds to the concern is the observation that these pathogens have been co-isolated in several patients. By examining the sputum of 21 cystic fibrosis patients, [13] found that in 5 of these cases, they were able to co-isolate *P. aeruginosa*, *S. aureus*, and members of the *B. cepacia* complex. This discovery underscores the potential for concurrent infection by all three species [13]. In a more extensive study conducted by Granchelli *et al.* (2018), involving 28,042 CF patients, these three species were found to be co-isolated in a substantial proportion of cases [14].

The cystic fibrosis (CF) environment plays a central role in the development of infectious biofilms, and tailored culture media have been formulated to mimic the chemical makeup of CF sputum. These

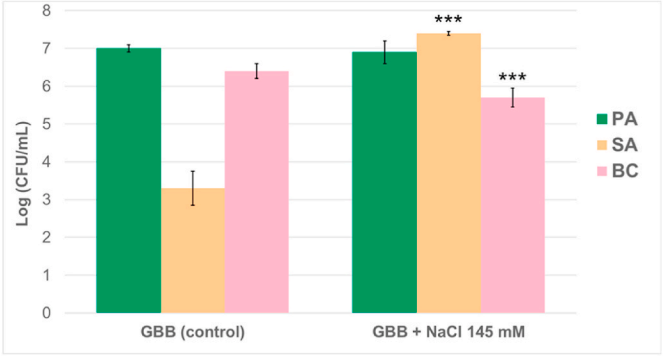

Fig. 7. CFU quantification after 48h of PA/SA/BC three-species biofilms. Inoculum ratio PA/SA/BC 1:500:1 in GBB or GBB supplemented with 145 mM NaCl (mean ± standard deviation; n = 2*3). ***p < 0.001.

media include essential components like amino acids, DNA, and mucin [32–35]. Among the chemical alterations observed in the airway surface liquid (ASL) of CF patients, the elevation of NaCl levels has emerged as a pivotal factor driving the development of biofilms within the ASL [2,42]. Building upon this insight, the current study seeks to explore the impact of NaCl on the growth of biofilms formed by PA, SA, and BC, both individually and in combination.

Building on prior research, we devised culture conditions to facilitate the concurrent growth of biofilms involving the three bacteria under scrutiny. These conditions were predicated on a restrained inoculum, utilizing a minimally composed medium enriched with glucose (GBB). The selection of glucose was predicated on its capacity to induce the upregulation of the *pslA* gene, a pivotal factor in PA biofilm formation [43]. Our approach involved intentionally minimizing or eliminating planktonic growth through strategies such as implementing new nutrient inputs, removing planktonic cells through medium renewal at 4-h and 24-h intervals. This strategic design aimed to foster biofilm development over the proliferation of planktonic populations [36,37]. Through these efforts, we achieved biofilms comprising adhered populations exceeding 6 Log (CFU/mL) for each bacterium, encompassing both reference strains and CF clinical isolates. These outcomes were realized after 48 h of incubation in GBB (Supplementary Fig. 2).

Our initial focus centered on PA/SA communities, driven by SA's capability to flourish in highly concentrated NaCl media [29]; [44]. Additionally, the widespread presence of PA further underscored the relevance of investigating this pair [45,46].

In a recent comprehensive review exploring the intricate dynamics between PA and SA, and delving into a thorough examination of *in vitro* co-culture experiments involving these two species, the authors emphasized the necessity of accounting for the impact of the microenvironment and strains with distinct genetic backgrounds. This emphasis on considering such factors becomes pivotal in the study of interactions between PA and SA [11].

Initial findings from the dual-species PA/SA (1:1) co-culture in GBB unmistakably demonstrated a competitive interaction between the two reference strains. It also underscored the challenge faced by SA in sustaining its presence within mixed biofilms. In contrast, the PA population exhibited stability akin to that seen in one-species biofilms. These observations align with prior research that has consistently indicated the tendency for PA to outcompete SA under simultaneous and equivalent ratio inoculations [47,48]. In pursuit of a well-balanced mixed biofilm, it was imperative to maintain a PA-SA ratio of at least 1:500, in line with findings from a preceding investigation [49].

Considering the classical sequence of lung infection in the CF context [31,50], we devised an experiment wherein 48-h-old SA biofilms were inoculated with a PA suspension. The outcomes of introducing PA onto pre-existing SA biofilms distinctly highlighted the antagonist nature of PA, leading to a notable reduction of 3 logs in the adherent SA population (Fig. 5). This pattern reveals that SA initially establishes growth within the biofilm but is subsequently outcompeted by PA. This phenomenon aligns with observations made by Woods *et al.*, who similarly noted a 2-log reduction in viable SA cells 24 h after introducing PA onto 5-day-old SA biofilms [51]. The findings from our qPCR analysis strongly imply that the SA population comprises cells that are either non-viable or non-culturable. Consequently, we corroborated that PA has the capacity to supplant a well-established SA biofilm (as indicated by CFU counts), even when the initial ratio favors SA.

As observed in prior *in vitro* and *in vivo* investigations, this behavior can be attributed to metabolic adaptations in both SA and PA, stemming from the competition for nutrients [48,52,53], but also of cooperation between the two species [85]. Recent studies also reported that transcriptomic modifications occur and affect the motility and virulence of PA [49] as well as SA amino acid and carbohydrate metabolisms, cell division [49], and Quorum Sensing (QS) systems [54,55]. Quorum Sensing, in particular, is widely acknowledged to exert a significant role in the interactions between PA and SA. Notably, PA is recognized for

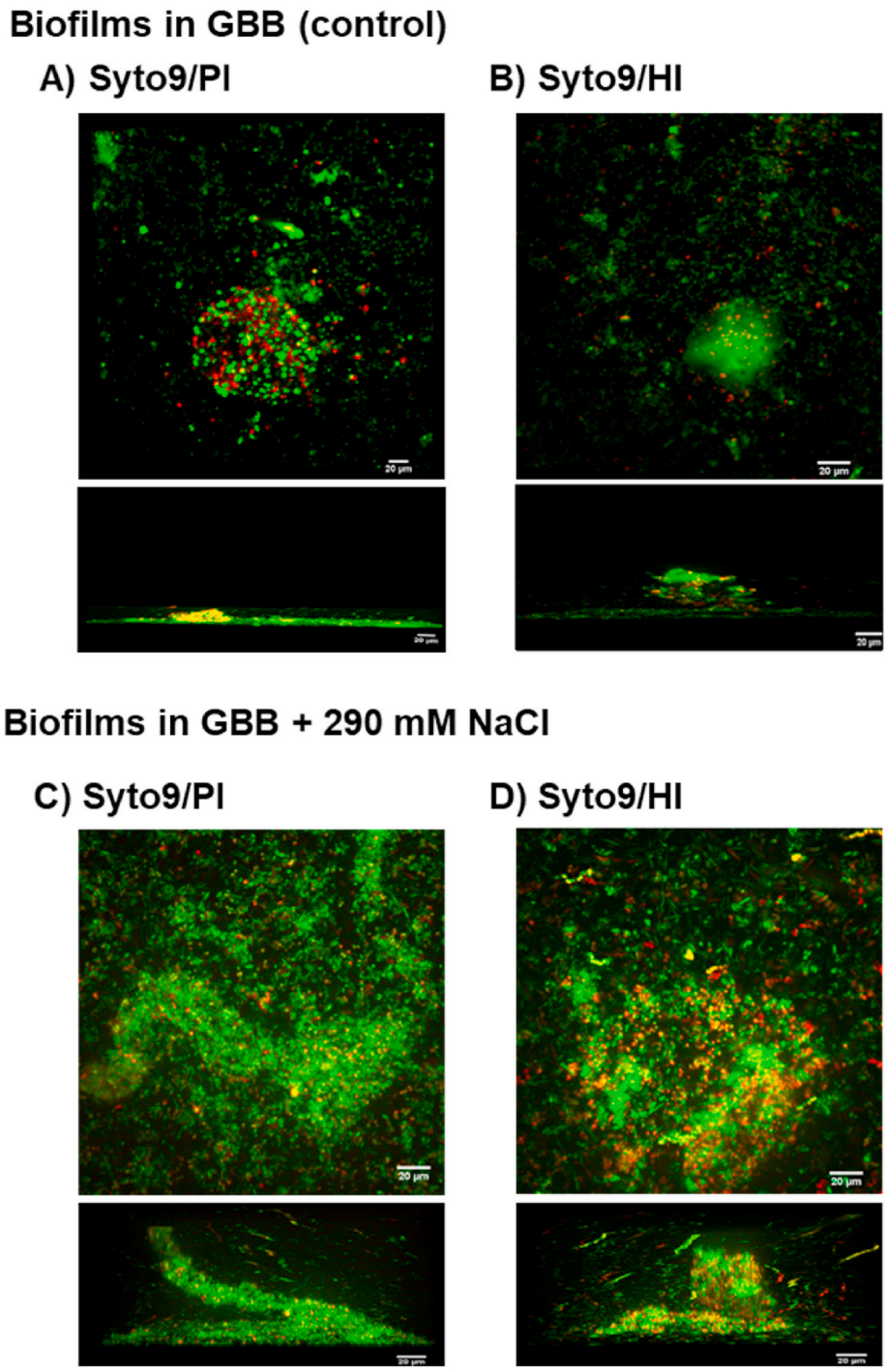

Fig. 8. CLSM images of PA/SA/BC three-species biofilms after 48h in GBB or GBB supplemented with 290 mM NaCl (Inoculum ratio 1:500:1). Cells were stained with A) and C) Syto9/PI for Live/Dead differentiation, and **B) and D)** Syto9/HI for Gram-/Gram+.

producing various compounds that impact *Staphylococcus* species. Among these, several molecules associated with the pqs QS system are noteworthy, including siderophores for competing in iron uptake, such as pyocyanin, which can inhibit SA's oxidative respiration. Additionally, there are proteases like LasA, capable of lysing SA, along with elastase and rhamnolipids, inducing biofilm dispersal, and 2-*n*-heptyl-4 hydroxyquinoline N-oxide (HQNO) [51,56,57].

When PA and SA grow together, several PA QS genes (*lasI*, *lasR*, *rhlI*, *rhlR* and *pqsH*) are upregulated while some SA QS genes (*sarA*, *sigB*) are downregulated, with the exception of *agrB*, which probably explains why SA is not completely killed [51]. In SA, the *agr* system controls SA attachment and biofilm development. It is also involved in virulence factors expression and in the ability of SA to disperse from other communities by stimulating the dispersal factors such as proteases and phenol-soluble modulins (PSMs) [58,59].

Grandjean Lapierre *et al.* (2017) conducted a study that shed light on the significance of airway salt concentrations among the multiple potential factors influencing bacterial colonization and subsequent infections in individuals with cystic fibrosis (CF), and propose the integration of salt concentration measurements into the standard assessment of CF respiratory tract samples. Although the precise sodium chloride (NaCl) concentration levels may differ during infections, their

research underscores the pivotal role of salt concentrations in shaping bacterial dynamics within the pulmonary environment [60].

Thus, based on the salt composition of CF sputa previously reported [33,60], we investigated three NaCl concentrations in supplementation of the GBB minimal medium (72.5 mM, 145 mM, and 290 mM). The 145 mM median concentration corresponds to the NaCl volume transport through the epithelial layer [39].

SA osmotolerance, with respect to high NaCl concentrations, has been widely investigated on both planktonic and biofilm populations, and attributed to the transcriptional sigma factor, σB, and the rbf and *icaA* genes, widely conserved among staphylococci [30,44,61]. In this study, we demonstrated the osmotolerance of SA, manifesting as a consistently stable adhered biomass up to 1.7% NaCl (w/v) equivalent to 290 mM. Likewise, we observed a noteworthy beneficial impact of 1.7% NaCl (w/v) on the adherent PA population. This concurs with existing research highlighting PA's halotolerance, which extends up to 5% (w/v) NaCl, both in isolation and when co-cultured with SA [45,46,62]. Remarkably, interventions employing elevated hypertonic saline conditions (ranging from 5.85% to 7.0% w/v) exhibited a positive impact on lung function and the frequency of pulmonary exacerbations [63–66].

We also extended our investigation to include CF clinical isolates, aiming to explore how elevated NaCl levels might influence their capacity to develop biofilms. Specifically, we focused on a mucoid strain of *P. aeruginosa* (mPA), known for its overproduction of alginate, a trait believed to facilitate biofilm formation [67,68]. The biofilm formed by mPA exhibited a lower count of adherent cells compared to the PAO1 reference strain. It's important to note that the utilization of a minimal medium and operating under pseudo-static conditions does not encourage the development of a PA biofilm at the air-liquid interface, as is commonly observed [69].

Concerning the biofilm-forming capability of SA clinical strains, the adherent population of MRSA surpassed that of both MSSA and the SA methicillin-resistant laboratory strain. This finding reaffirms the influence of genetic background on the propensity to develop biofilms [70,71]. Regarding two-species biofilms, initial findings indicated that both MRSA and MSSA could coexist and thrive alongside the mucoid PA. This observation aligns with prior research that highlighted how the mucoid phenotype of PA promotes its coexistence with SA [68,72]. As noted by O'Brien and Fothergill, the transition to mucoidy is frequently linked to reduced virulence of PA, both *in vitro* and *in vivo* [57].

In the presence of 145 mM NaCl, a notable rise in mPA populations was evident within the two mixed biofilms. However, an increase in the SA population was specifically observed for the MSSA strain, which was isolated from the same patient. Notably, across the evaluated range of NaCl concentrations, we did not observe any antibacterial activity or adverse effects on biofilm formation. This was further confirmed by the absence of a substantial increase in damaged or dead cells, as indicated by PI staining, even at higher NaCl concentrations. Therefore, the pivotal outcome from the PA/SA biofilm investigation is the substantial enhancement of SA's colonization within mixed communities under high NaCl levels, bolstering its resilience against PA's antagonistic behavior. Concurrently, PA adherent populations also demonstrated gradual increments.

Subsequently, we proceeded to assess the capacity of *B. cepacia* to grow independently and as part of two-species biofilms alongside PA and SA. As far as our knowledge extends, the impact of NaCl on *Burkholderia* has only been documented for *B. cenocepacia* and *B. pseudomallei*. It's worth noting that, in those instances, no discernible alteration in growth rate was observed up to a NaCl concentration of 2.6% (w/v) [73], an increase in expression of several virulence factors under NaCl stress has been reported [73–75]. In our experiments, we found that NaCl had no discernible impact on pure BC biofilms.

When cultured alongside SA in GBB, BC demonstrates dominance, yet does not entirely eliminate SA, aligning with findings from prior investigations [76,77]. The decline in the adherent SA population in the presence of BC could potentially be attributed to membrane damage resulting from enzymatic peptidoglycan lysis executed by BC [78]. Conversely, BC and PA exhibit a tangible ability to coexist and thrive in tandem, a phenomenon potentially elucidated by BC's competence to utilize and react to PA's Quorum Sensing signals [79,80].

Lastly, we also scrutinized the influence of 145 mM NaCl on a polymicrobial consortium comprising PA/SA/BC, comparing it with their respective monospecies counterparts. Remarkably, we noted an augmentation in total biomass in comparison to the three-species biofilm devoid of NaCl, even though the composition of the biofilm was substantially altered in terms of species distribution. At elevated NaCl concentrations, SA emerges as the predominant species, affirming the beneficial impact of NaCl on SA within mixed biofilms, while the PA population remains constant. Simultaneously, a notable reduction of 1.86 Log was observed for the BC adherent population. These outcomes unequivocally underscore that the solitary NaCl parameter holds the capacity to modulate species distribution in static polymicrobial biofilms.

In summary, this study sheds light on the intricate interplay between microenvironments and polymicrobial communities comprising three species that are often (co)-isolated in the context of CF infections. To the best of our knowledge, it marks the inaugural attempt to delve into the influence of NaCl concentration on the establishment of mono-to three-species biofilms encompassing *P. aeruginosa*, *S. aureus*, and *B. cepacia*. While acknowledging that these conditions may not perfectly replicate the complexity of the CF environment, the significant role of heightened NaCl concentrations in fostering and reshaping multi-species biofilms *in vitro* strongly implies its pivotal involvement in lung colonization within CF patients, as well as infection outcomes. A promising avenue of future exploration will revolve around probing the adaptive responses of polymicrobial biofilms within these distinctive conditions.

4. Methods

4.1. Bacterial strains

Pseudomonas aeruginosa PAO1 wild type was obtained from the Institute Pasteur Collection (CIP 104116, Paris, France). Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC® 33591™) and Burkholderia cepacia (ATCC® 25416™) were both obtained from the ATCC® (Manassas, USA). Clinical strains: one mucoid *P. aeruginosa* and two *S. aureus* strains: one methicillin-sensitive (MSSA) and one methicillin-resistant (MRSA), were from the Collection of Purpan hospital (Toulouse, France), isolated from CF patients on cetrimide and mannitol salt agar, respectively (Table 2). Identification was performed regarding macroscopic and microscopic characteristics and biochemical properties (oxidase, catalase). Finally, strains were identified at the species level by MALDI-TOF MS (Maldi Biotyper Microflex®, Bruker Daltonics, Bremen, Germany; IVD 7712) (score $\geq$2.2). In the present study, the bacterial strains will be referred to as follows:

Strains were frozen and kept at −80 °C in a 20% (v/v) glycerol stock solution. Before each experiment, two successive subcultures were prepared on trypticase soy agar (TSA, Biomérieux, Craponne, France) and incubated for 24 h under aerobic conditions at 37 °C. Before each experiment, 10^8 CFU/mL bacterial suspension was prepared by adjusting OD at 640 nm to 0.150.

4.2. Media

The minimal culture medium used for the evaluation of multi-species biofilm formation was selected regarding the composition of media previously defined for PA and SA monospecies biofilms, respectively MBB and BB [36,37] (Supplementary data). A first step studied the biofilm formation of each species alone (PA, SA and BC, respectively) in MBB and BB media. The new minimal medium, named Glucose-supplemented Biofilm Broth (GBB, 1X), was used. GBB has the same composition as BB, with supplementation with D-glucose (0.05

g/L). The pH of the GBB medium was 7.26 ± 0.03.

4.3. Biofilm formation

One-species biofilms were grown in 24-well microtiter plates for 48 h. The culture medium was composed of 900 µL of GBB_{2X} and 1.0 mL distilled sterile water. The plate was inoculated with 100 µL of a 10^2 CFU/mL (10 CFU/well) bacterial suspension of *P. aeruginosa*, *S. aureus* or *B. cepacia*, derived from a 10^8 CFU/mL suspension in the corresponding 2X medium through serial dilutions. For all experiments, CFUs numeration was controlled on the prepared suspension. Then, the microtiter plate was incubated at 37 °C, under aerobic conditions. During incubation, each well was emptied and rinsed twice with distilled sterile water, and the culture medium was renewed after 4 h and 24 h of culture. The enumeration of adhered bacteria was carried out as previously described [81]. In brief, at 4 h, 24 h, and 48 h of culture, the medium was aspirated, and each well was rinsed twice using 2.0 mL of sterile distilled water. Subsequently, 1.0 mL of sterile distilled water was introduced to the microtiter plate, and the bottom of each well was gently scraped with a sterile spatula. The content of each well was collected, and serial dilutions were prepared. Next, 100 µL from each dilution was spread onto TSA plates, which were then incubated at 37 °C for 24 h. Following incubation, the Colony Forming Units (CFUs) were enumerated, and the concentration was calculated in terms of Log (CFU/mL).

For two- and three-species biofilm formation, the volume of GBB_{2X} medium was adapted, depending on the volume of bacterial suspension. For two-species biofilms, 800 µL GBB_{2X} were added to 100 µL of each suspension and, for three-species biofilms, 700 µL GBB_{2X} was added to 100 µL of each of the three suspensions. Thus, since suspensions were prepared in culture medium, the final volume of GBB_{2X} was constant (1.0 mL), and 1.0 mL of sterile distilled water was added to it. PA and BC were always inoculated with a 10^2 CFU/mL suspension, while various inoculum concentrations of SA, from 10^2 CFU/mL to 5.10^4 CFU/mL, were assayed (10 to 5.10^3 CFU/well). After inoculation, the microtiter plate was incubated at 37 °C for 48 h. The culture medium was also renewed at 4 h and 24 h of incubation and biofilm sampling was performed in the same way as for one-species biofilm formation. In order to quantify each population individually, SA CFU were counted on Mannitol Salt Agar medium, and PA and BC CFU numeration was carried out on TSA, based on their different morphology. The utilization of the crystal violet (CV) staining assay was deemed unsuitable due to its non-specific characteristics, which prevent the differentiation of species within polymicrobial communities [82].

In the case of PA/SA two-species biofilms, the competitive index (CI) was computed using the following formula, where "Log" represents the Log (CFU/ml):

$$CI = \frac{\text{Log SA}_{final}/\text{Log PA}_{final}}{\text{Log SA}_{initial}/\text{Log PA}_{initial}}$$

4.4. Exploration of NaCl concentration effect on adhered populations

Three distinct NaCl concentrations were assessed: 72.5 mM, 145 mM, and 290 mM. To create solutions with double concentration (2X), sterile distilled water was utilized. In each well, except for the control, 1.0 ml of the NaCl solution (2X) was introduced. Alternatively, in the control well, 1.0 ml of sterile distilled water was added. The NaCl used was procured from Sigma-Aldrich (Saint-Quentin Fallavier, France).

4.5. Sequential inoculation of S. aureus and P. aeruginosa

SA biofilms were cultivated in a 24-well microtiter plate using the GBB medium for a duration of 48 h. The microplates were arranged following the single-species biofilm procedure, incorporating medium renewals at 4 h and 24 h into the incubation, as previously outlined. Once the 48-h period was reached, the microplate wells underwent two rinses with sterile distilled water, after which the culture medium was refreshed. Wells designated for two-species biofilms were subsequently subjected to inoculation with 100 µL of a suspension containing 10^2 CFU/mL of PA. Following PA inoculation (t = 72 h), the medium was renewed after an additional 24 h. At the culmination of 48 h post-PA inoculation, the process of biofilm sampling and quantification was conducted in line with the protocol delineated for the two-species biofilm. Control wells encompassing SA were utilized to assess the SA population prior to PA introduction (designated as control t0), while another control was maintained throughout the entire duration of the experiment (designated as control 48 h). Additionally, a separate control experiment, lasting 48 h and involving PA alone, was also executed.

4.6. Confocal Laser Scanning Microscopy (CLSM)

CLSM acquisitions were performed for qualitative purposes regarding the ratio of viable/damaged cells (Syto9/IP) and the distribution of SA within the biofilm (Syto9/HI).

Biofilms were grown in a 6-well plate, by adjusting the 24-well assay proportions. Observations were made at the Fédération de la Recherche Agrobiosciences Interactions et Biodiversité (FRAIB) with a Perkin Elmer Ultraview spinning disk microscope equipped with a x60 immersion objective (Nikon) and an EM_CCD camera (Hamamatsu). Biofilms were stained with fluorescent labelling Syto9 and propidium iodide (PI) to distinguish live/dead bacteria (0.5 µL was necessary for each marker). For Gram-positive/Gram-negative differentiation, Syto9 and hexidium iodide (HI) stains were used. Signals were recorded in the green channel (excitation 488 nm, emission 500–540 nm) for Syto9 and in the red channel (excitation 561 nm, emission 553–623 nm) for both PI and HI. The staining products were purchased from ThermoFisher Scientific (Illkirch, France). Furthermore, for each sample observed using CLSM (1 well of 6-well microplate), at least 3 acquisitions were made to obtain a more accurate representation of the whole biofilm. The reported images were the most representative of the well. Images were acquired using the Volocity software and processed with ImageJ (1.53a). The relative quantification of the fluorescence from Syto9/PI and Syto9/HI stains was calculated for the red and green fluorescence, respectively and reported in Table S1.

4.7. qPCR analysis

Bacterial lysis and DNA extraction were performed for Gram-negative and Gram-positive bacteria on 200 µL or 1.0 mL samples, respectively, obtained after microplate scraping. This step was carried out using the QIAamp DNA Mini Kit protocol (Qiagen, France).

The primer sequences are listed in Supplementary Table 1. Two different primers were used for these experiments: primers of the *P. aeruginosa* rhlR gene and a primer of the *S. aureus femA* gene. The design of *P. aeruginosa* PAO1 *rhlR* primers was previously performed by Ref. [83] using the Primer Express software [v.3.0] [83].

S. aureus quantification was performed using the primers employed by Francois *et al.* [84]. They designed primers for the *femA* gene, coding for aminoacyltransferase FemA and partly responsible for the methicillin-resistant phenotype of Staphylococcus. To ensure the specificity of the primers, their sequences were first subjected to a BLAST analysis on the genome of the bacteria carrying the gene from which primers were designed. A similar analysis was carried out on the genome of the other bacteria studied (PA, SA and BC). Further analysis concerning guanine and cytosine contents, melting temperature, and homo- and heterodimer formation were carried out using the online software OligoAnalyzer [© 2019 Integrated DNA Technologies, Inc.].

DNA quantification used 12.5 µL of the QSYBR green supermix (Bio-Rad, France) complemented with 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM) and 6.5 µL of elution buffer. Finally, 5.0 µL of DNA sample from both the microplate and the calibration range experiments (know bacterial concentrations from 102 to 108 CFU/mL

prepared from subcultures on TSA and suspended in PBS), were added. For all qPCR experiments, three technical replicates and three independent assays were performed (n = 3x3). The minimal R2 obtained was 0.99 for both evaluated species. Each analysed sample was quantified at least in triplicate. qPCR was then performed in an IQ5 thermocycler (Bio-Rad, France). The Gram-negative quantification program was set as follows: initial denaturation: 3 min, 95 °C; 50 runs to visualise fluorescence and threshold values well; CT: denaturation: 10 s, 95 °C; hybridisation: 30 s, 60 °C and elongation, 30 s, 72 °C. Melting curves were also analysed: 1 min, 95 °C + 1 min, 55 °C. Then a rise in temperature from 55 °C to 95 °C allowed the temperature characteristic of the amplification to be identified and, thus, the specific binding of the primers to be verified [83]. The Gram-positive quantification was performed using the following cycling parameters: 2 min at 50 °C followed by 10 min at 95 °C; denaturation: 15 s, 95 °C (50); hybridisation: 1 min at 60 °C (50) and elongation: 30 s, 72 °C (50) [84]. Melting curves were also drawn as previously detailed. Results were expressed as Genomic Units (GU).

4.8. Statistical analysis

For each experiment with CFU counts, two technical replicates were performed on two distinct wells. Independent assays were performed at least three times for all experiments (n = 2x3 or n = 2x4) except for assays on referenced strains for the selection of a Minimal Medium and for assay on two-species biofilms with NaCl using the same inoculum (1:1) (two independent assays; n = 2x2) (Supplementary data). For qPCR analysis, 3 technical replicates were performed in three independent assays (n = 3x3). All statistical analyses were performed using R-studio interface [v1.2.5001]. In order to evaluate the difference between two samples, a Student-t-test was used, after verification of data normality via a Shapiro-Wilk test. Whenever normality was not demonstrated, a Wilcoxon-Mann-Whitney test was carried out. Statistically significant values were defined as p-values of 0.05 (*), 0.01 (**), or 0.001 (***). Specific statistical analysis comparing Log (CFU/mL) and GU data (Fig. 3) were also represented as follows: $p < 0.05$ (°); $p < 0.01$ (°°); $p < 0.001$ (°°°).

Credit author statement

F.E.G: conceived and supervised the study. C.R: conceived the study. J.T: carried-out the experiments, statistical analysis, and redaction of the first draft of the manuscript. J.T: carried out the microscopy analyses. M. R: carried out the microscopy analyses. J.T: contributed to conception and planning the experiments, to results interpretation, as well as redaction and revisions of the manuscript. C.R: contributed to conception and planning the experiments, to results interpretation, as well as redaction and revisions of the manuscript. F.E.G: contributed to conception and planning the experiments, to results interpretation, as well as redaction and revisions of the manuscript. M.R: participated to discussion and results interpretation. B.L: participated to discussion and results interpretation. All authors contributed to manuscript revision, read, and approved the submitted version.

Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Fatima El Garah reports financial support was provided by Regional Council of Occitanie.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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