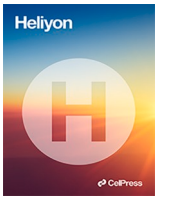
Heliyon 9 (2023) e21469

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Effect of glucose on growth and co-culture of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in artificial sputum medium

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ARTICLE INFO

Keywords:
Artificial sputum medium
Glucose
Pseudomonas aeruginosa
Staphylococcus aureus
Co-culture

ABSTRACT

People with cystic fibrosis-related diabetes (CFRD) suffer from chronic infections with *Staphylococcus aureus* and/or *Pseudomonas aeruginosa.* In people with CFRD, the concentration of glucose in the airway surface liquid (ASL) was shown to be elevated from 0.4 to 4 mM. The effect of glucose on bacterial growth/interactions in ASL is not well understood and here we studied the relationship between these lung pathogens in artificial sputum medium (ASM), an environment similar to ASL *in vivo.*

S. aureus exhibited more rapid adaptation to growth in ASM than *P. aeruginosa*. Supplementation of ASM with glucose significantly increased the growth of *S. aureus* ($p < 0.01$, $n = 5$) and *P. aeruginosa* ($p < 0.001$, $n = 3$). ASM conditioned by the presence of *S. aureus* promoted growth of *P. aeruginosa* with less lag time compared with non-conditioned ASM, or conditioned medium that had been heated to 121 °C. Stable co-culture of *S. aureus* and *P. aeruginosa* could be established in a 50:50 mix of ASM and *S. aureus*-conditioned supernatant.

These data indicate that glucose, in a nutrient depleted environment, can promote the growth of *S. aureus* and *P. aeruginosa.* In addition, heat labile factors present in *S. aureus* pre-conditioned ASM promoted the growth of *P. aeruginosa*. We suggest that the use of ASM allows investigation of the effects of nutrients such as glucose on common lung pathogens. ASM could be further used to understand the relationship between *S. aureus* and *P. aeruginosa* in a co-culture scenario. Our model of stable co-culture could be extrapolated to include other common lung pathogens and could be used to better understand disease progression *in vitro.*

1. Introduction

Staphylococcus aureus and *Pseudomonas aeruginosa* are both opportunistic pathogens that are known to cause severe respiratory infections in humans. While these pathogens do not commonly cause infections in healthy individuals, they are often found in people with chronic lung diseases, such as Cystic Fibrosis (CF), and can persist in the lungs for several years despite antibiotic treatment, by utilising their own genomic and metabolic changes [1]. *S. aureus* is found in the lungs of 70 % of CF patients in the 10-20-year-old age group, decreasing steadily to approximately 25 % in 60-year-olds, while *P. aeruginosa* is found in approximately 70 % of CF patients by the time they reach 30 years of age [2]. Data from the CF database [2] indicates that there are periods where *S. aureus* and *P. aeruginosa*

Abbreviations: ASL, airway surface liquid; ASM, artificial sputum medium; CF, cystic fibrosis; CFRD, cystic fibrosis related diabetes.

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https://doi.org/10.1016/j.heliyon.2023.e21469
Received 12 June 2023; Received in revised form 5 October 2023; Accepted 21 October 2023
Available online 23 October 2023

co-exist in the lungs, however, *P. aeruginosa* eventually outcompetes *S. aureus*. It is much harder to estimate the prevalence of *S. aureus* and *P. aeruginosa* in the lungs of people with other respiratory diseases, such as chronic obstructive pulmonary disease (COPD), due to the lack of extensive longitudinal clinical data. Various studies utilising sputum samples, bronchoalveolar lavage (BAL) and protective specimen brushings (PSB) from COPD patients demonstrated the occurrence of *S. aureus* in 1–40 % of all COPD patients [3–6], while the prevalence of *P. aeruginosa* in COPD patients ranged from 4 % to 34 % and presence of both pathogens was associated with the severity of the condition, frequency of readmissions and frequency of exacerbations [7–10].

In people with CF, variations in the cystic fibrosis transmembrane conductance regulator (CFTR) result in impaired airway bicarbonate and fluid secretion, producing a dehydrated airway surface liquid (ASL) and viscous mucous [11–13]. This causes the impairment of the mucociliary clearance mechanisms resulting in an inability to remove bacteria from the environment which contains amino acids, mucins and other sources of nutrients [14]. Such changes contribute to the increased risk of acquiring lung pathogens. A subset of CF patients who develop diabetes (CF-related diabetes; CFRD) are proposed to be at higher risk of their lungs being colonised by *P. aeruginosa* and *S. aureus* [15–17,18]. Once established, *P. aeruginosa* becomes difficult to eradicate from the lungs as it has the capacity to rapidly develop resistance to antibiotic therapy [19].

Previously it was demonstrated that the glucose concentration in ASL is approximately 0.4 mM in healthy individuals, however, it was elevated in people with CF to ~2 mM, and was further elevated to ~4 mM in those with CFRD [18]. These findings were replicated *in vitro* using airway epithelial cells grown at air-liquid interface, to form a differentiated epithelial layer, and exposed to normo- and hyperglycaemic media and *in vivo* using wild type and hyperglycaemic mice [20–22]. Thus, glucose could provide a nutrient for growth of these respiratory pathogens. However, these are challenging models in which to study the effects of glucose on the growth and co-existence of these organisms due to the complexity of the systems involved. Other studies have investigated the relationship between these bacteria *in vitro* using typical bacterial growth media such as tryptic soy broth and lysogeny broth [23–27]. These media ensure good bacterial growth by providing an extremely nutrient-rich environment which does not represent the conditions in the lung. Furthermore, it is difficult to investigate the specific effects of glucose on bacterial growth using such media because of the abundance of other available nutrients [28]. Artificial sputum medium (ASM) is a culture medium that was designed to mimic sputum from CF patients. It contains similar components to CF sputum, such as amino acids, mucin and DNA [29]. ASM was originally designed to test antibiotic efficacy against *P. aeruginosa.* It was shown that *P. aeruginosa* growth in ASM is similar to growth in the lungs of CF patients [30,31]. Therefore, our aims were first to develop an ASM model to study the effect of glucose on the growth of *S. aureus* and *P. aeruginosa* in monoculture and in co-culture as an *in vitro* environment more relevant to the CF lung. Our second aim was to see if we could establish a stable co-culture of *S. aureus* and *P. aeruginosa* as an *in vitro* model of co-existence in the CF lung. We hypothesised that the presence of glucose in ASM would promote the growth of *S. aureus* and *P. aeruginosa* and change the interaction of these bacteria in co-culture.

2. Materials and methods

2.1. Strains of P. aeruginosa and S. aureus

Staphylococcus aureus (ATCC29213) and *Pseudomonas aeruginosa* (H174) were used. The H174 strain of *P. aeruginosa* is a modified PAO1 strain with inserted *lux* genes. These genes allow bacteria to continuously produce light as a by-product of metabolism [32]. Measuring emitted light allows for precise real-time quantification of viable bacteria.

2.2. Quantification of live bacteria

To enable quantification of *P. aeruginosa* and *S. aureus* in Muller Hinton (MH) medium, ASM and when in singular or co-culture, two different approaches were used. *P. aeruginosa* was quantified by measuring luminescence produced by bacteria using a Tecan microplate reader (ThermoFisher Scientific). 200 μl of culture was added to a well of a white flat-bottomed 96-well plate (Thermofisher Scientific). Background luminescence in the absence of culture was subtracted. Sample Colony Forming Units (CFU) were resolved using plots of luminescence against standards of CFU per millilitre (over the range utilised in these experiments 10^5–10^9) and obtained using the Miles and Misra approach. *S. aureus* was quantified using the Miles and Misra approach [33]. Bacterial culture was serially diluted in sterile PBS (Sigma-Aldrich) and plated onto mannitol salt agar (MSA) plates. Plated bacteria were incubated at 37 °C for 24 h and the colonies formed were then counted. Final CFU/ml was calculated using equation CFU/ml = (colony count x dilution factor)/volume of culture plated. During the initial experimental setup culture purity of *P. aeruginosa* and *S. aureus* colonies and selection of *S. aureus* on MSA were confirmed using MALDI-TOF Mass Spectrometry due to the absence of antibiotics in the media and plates.

2.3. ASM preparation

ASM was prepared as described in Sriramulu DD, [34]. 1 L ASM contained 5 g of pig mucin (type 2), 4 g of low molecular weight salmon sperm DNA, 5.9 mg of DTPA, 5 g of NaCl, 2.2 g of KCl, and 1.81 g of Tris base, 5 g of casamino acids and adjustedto pH 7.0. ASM was sterilised at 121 °C for 15 min. After sterilisation, 5 ml of egg yolk emulsion was added under sterile conditions. ASM was then aliquoted into 50 ml tubes and kept at 4 °C for up to 4 weeks.

2.4. ASM inoculation with bacteria

Initial preparation of ASM consisted of 15 ml media in a 50 ml tube warmed up to room temperature. Subsequently, bacteria were added to approximately 3×10^5 live CFU/ml and cultures were incubated at 37 °C with constant shaking at 600 RPM. To create a ASM hyperglycaemic environment, glucose was added to 4 mM final concentration prior to the addition of bacteria. Glucose is always added at the initial stage of inoculation unless stated otherwise.

2.5. Preparation of supernatant and heat-treatment of supernatant

To collect bacterial supernatant, ASM with bacteria was centrifuged at 7000 g at 4 °C for 15 min to pellet the bacterial cells. The supernatant was transferred to a new sterile 50 ml tube and the pellet was discarded. The 'boiled' supernatant was produced by autoclaving at 121 °C for 15 min. No additional glucose was added at the supernatant generation step unless otherwise stated.

2.6. Measurement of glucose concentration

Glucose utilisation by bacteria in the ASM was quantified by the glucose oxidase method using the Analox Glucose Analyzer (Analox Instruments) according to the manufacturer's guidelines. For the glucose measurement, culture supernatant was used, rather than a pure culture, to avoid contamination of the machine.

2.7. Measurement of lactate concentration

Lactate production in the ASM was measured using Lactate Assay Kit MAK064 (Sigma-Aldrich) following the manufacturer's guidelines. ASM supernatant was used for the lactate measurement.

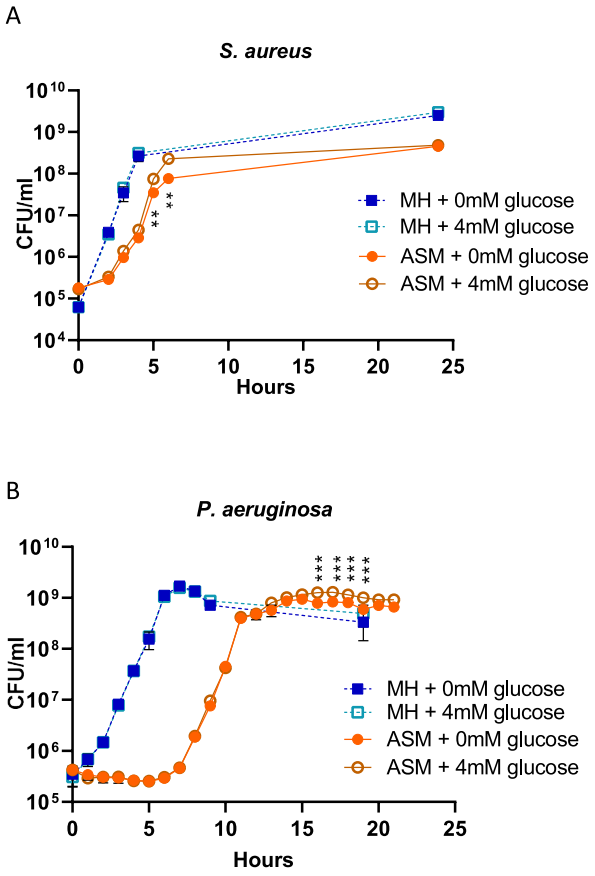

Fig. 1. Growth of *S. aureus* and *P. aeruginosa* in MH and ASM with and without 4 mM glucose. (A) Growth of *S. aureus* and (B) growth of *P. aeruginosa* in MH (n = 3) and ASM (n = 5) in the absence (0 mM) or presence (4 mM) glucose over a 24-h period. Presented as mean ± SD CFU/ml. Effect of glucose was compared using repeated measures two-way ANOVA with Geisser-Greenhouse correction Significantly different from growth in absence of glucose **P < 0.01, ***P < 0.001.

2.8. Statistical analysis

Differences in survival, bacterial load, or growth of *S. aureus* and *P. aeruginosa* in normoglycaemic and hyperglycaemic conditions were compared using repeated measures two-way analysis of variance (ANOVA) with Geisser-Greenhouse correction using GraphPad Prism 8. Pearson correlation coefficients were calculated to determine the correlation between glucose concentration in the media and bacterial growth.

3. Results

3.1. S. aureus and P. aeruginosa growth pattern changes in ASM compared to MH

S. aureus and *P. aeruginosa* exhibited different growth patterns in ASM and MH. In MH, *S. aureus* entered logarithmic growth almost immediately reaching 5×10^8 CFU/ml in just 4 h (n = 3). In ASM, *S. aureus* exhibited a more prolonged (2-h) adaptive lag phase after which it entered the logarithmic phase, reaching 7×10^7 CFU/ml after 6 h (n = 5) (Fig. 1A).

Like *S. aureus*, *P. aeruginosa* immediately began logarithmic growth in MH reaching 1×10^9 CFU/ml in 6 h, at which point CFU plateaued (n = 5). However, *P. aeruginosa* growth in ASM underwent an even more prolonged lag phase of 7 h (n = 3). (Fig. 1B).

Despite delayed onset of growth *P. aeruginosa* reached similar final CFU/ml concentrations in ASM to that of MH at 20–24 h. These data indicate that *S. aureus* adapts more quickly to the nutrient-poor media and can utilise available nutrients for growth, whereas *P. aeruginosa* requires several hours to begin active growth in ASM.

3.2. Glucose promotes the growth of S. aureus and P. aeruginosa in ASM, but not in MH

The addition of 4 mM glucose (maximal glucose concentration reported in ASL) into ASM significantly increased the growth of *S. aureus* ($p < 0.01$, n = 5) and *P. aeruginosa* ($p < 0.001$, n = 3) whilst the addition of glucose to MH had no effect (Fig. 1A and B). The maximal effect on *S. aureus* growth was detected at 4–6 h, during the exponential growth phase, where CFU/ml increased from 4×10^6 to 2.2×10^8 in glucose (a 55-fold increase, n = 5) compared to no glucose medium where CFU increased from 2.8×10^6 to 7.6×10^7 (27-fold increase, n = 5). The maximal effect of glucose on *P. aeruginosa* growth was observed much later, between 15 and 20 h and during the plateau phase. The final concentration of bacteria in ASM at 24 h was, *S. aureus* (4.5×10^8 in 0 mM glucose and 4.8×10^8 CFU/ml in 4 mM glucose, n = 5, Fig. 1A) and *P. aeruginosa* (6.5×10^8 in 0 mM glucose and 9.2×10^8 CFU/ml in 4 mM glucose, n = 3, Fig. 1B).

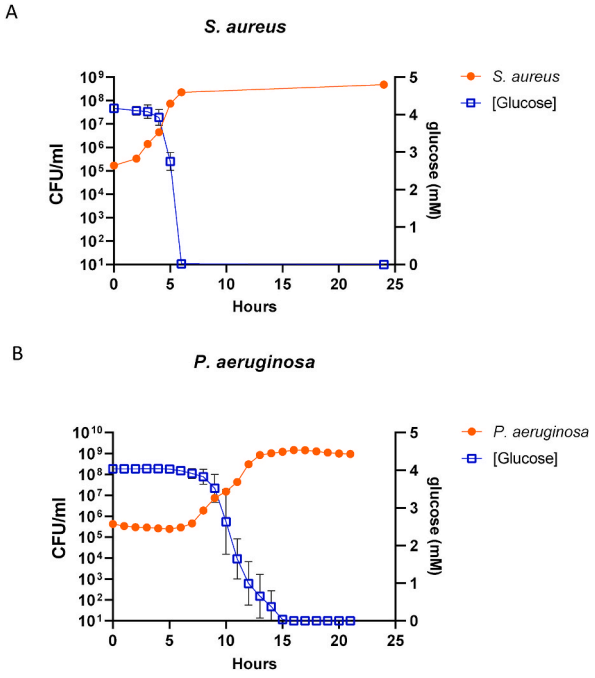

Fig. 2. Correlation between growth of *S. aureus* and *P. aeruginosa* and glucose depletion in ASM. Growth of (A) *S. aureus* or (B) *P. aeruginosa* in ASM and glucose concentration changes in the culture over 24 h. Presented as mean ± SD. Correlation between CFU/ml and glucose concentration (mM) was calculated using Pearson correlation coefficient; *S. aureus;* $p < 0.01$ *P. aeruginosa;* $p < 0.0001$.

3.3. S. aureus and P. aeruginosa deplete glucose in ASM

In ASM containing 4 mM glucose, the addition of *S. aureus* did not significantly decrease the glucose concentration in the first 4 h, while *S. aureus* increased from 1.5×10^5 to 4×10^6 CFU/ml. During the accelerated period of growth between 4 and 6 h, all the glucose in the media was utilised, CFU/ml increased from 4×10^6 to 2.2×10^8 and bacterial growth then plateaued (Fig. 2A). Pearson correlation coefficient showed that growth was dependent on glucose consumption ($R = 0.8343$, $p < 0.005$, $n = 5$, $Df = 6$). Similarly, during the lag phase of *P. aeruginosa* growth between 0 and 7 h, glucose concentration did not decrease. During the rapid growth between 7 and 13 h, *P. aeruginosa* increased from 4×10^5 to 8×10^8 CFU/ml and glucose concentration in the ASM decreased from 4 mM to ~0.65 mM and then became undetectable. After 13 h *P. aeruginosa* plateaued at approximately 1×10^9 CFU/ml (Fig. 2B). Pearson correlation coefficient showed that growth was dependent on glucose concentration ($R = 0.8528$, $p = <0.0001$, $n = 5$, $Df = 22$).

3.4. S. aureus initially outcompetes P. aeruginosa when co-cultured in ASM

In order to investigate the relationship between *S. aureus* and *P. aeruginosa* growth in normal and hyperglycaemic ASM environment, we attempted to create a stable co-culture by simultaneously adding both bacteria to ASM with and without glucose. When similar CFU of *S. aureus* and *P. aeruginosa* were added into ASM, the numbers of both bacteria decreased in the first 2 h. *S. aureus* growth recovered and at 22 h reached 9×10^7 CFU/ml in ASM after which growth plateaued. On the other hand, *P. aeruginosa* continued to decrease for up to 7 h and although growth then started to rise, numbers did not recover to starting CFU (Fig. 3A). Thus, the rapid adaptation of *S. aureus* to growth in ASM, plus the more rapid utilisation of glucose (Fig. 2) potentially provided a growth advantage over *P. aeruginosa* in co-culture.

3.5. P. aeruginosa outgrows S. aureus in co-culture in pre-conditioned ASM

Current evidence suggests that *P. aeruginosa* infection is often secondary to *S. aureus* in the lungs [28]. Therefore, we obtained supernatant that had previously supported a culture of *S. aureus* for 24 h (pre-conditioned ASM). We then added both *P. aeruginosa* and

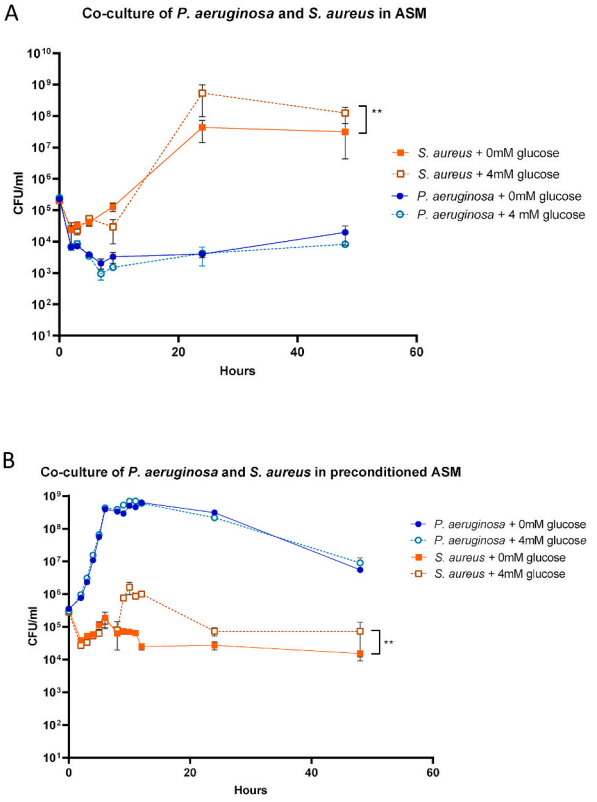

Fig. 3. Co-culture of *P. aeruginosa* and *S. aureus* in ASM or pre-conditioned ASM supernatant. A. Equal amounts of *S. aureus* and *P. aeruginosa* were added to ASM in the absence or presence of 4 mM glucose; B. pre-conditioned ASM supernatant (where glucose was added at supernatant generation stage) and cultured for 48 h. Presented as mean ± SD. Effect of glucose was compared using repeated measures two-way ANOVA with Geisser-Greenhouse correction. Significantly different, $^{**}p < 0.01$.

S. aureus into pre-conditioned ASM with either 0 mM or 4 mM glucose. In pre-conditioned ASM, *S. aureus* CFU initially decreased in the first 2 h. The CFU then increased more in media with 4 mM glucose compared with no glucose ($p < 0.01$, $n = 3$), before reaching steady levels from 24 to 48 h that were lower than starting CFU (Fig. 3B). Unlike growth in unconditioned ASM, the population of *P. aeruginosa* immediately entered logarithmic growth in pre-conditioned ASM. *P. aeruginosa* grew from 3×10^5 to 4×10^8 CFU/ml in just 6 h. Over 48 h, *P. aeruginosa* decreased to 5×10^6 CFU but remained higher than that of *S. aureus* (Fig. 3B). Thus, in the supernatant derived from *S. aureus* culture (pre-conditioned ASM), *P. aeruginosa* growth was initiated more rapidly and *S. aureus* numbers remained low, a reversal of that seen in ASM. This indicates that *S. aureus* adapts better to growth in ASM and the presence of glucose than *P. aeruginosa* but may produce factors that then enable better adaptation/growth of *P. aeruginosa* in ASM.

3.6. *P. aeruginosa outcompetes S. aureus when added to the established S. aureus culture in the supernatant*

In order to extend our first model and accommodate the pre-conditioned ASM findings, we allowed *S. aureus* to grow in ASM for 24 h, at which point we added 2×10^5 CFU/ml of *P. aeruginosa* into the media. Immediately after the addition of *P. aeruginosa*, both pathogens exhibited a rapid decline in CFU (Fig. 4A). In the first 2 h, *P. aeruginosa* decreased from 2×10^5 to 1×10^4 CFU/ml, while *S. aureus* decreased from 2×10^9 to 3×10^7 CFU/ml (Fig. 4A). It took 9 h for *S. aureus* to recover back to initial values, while the amount of live CFU of *P. aeruginosa* continued to decrease for 9 h. After this time, however, the growth of *P. aeruginosa* increased from 5×10^3 to 1×10^6 CFU/ml. Over the next 24 h *P. aeruginosa* continued to grow overtaking *S. aureus*. After 48 h in culture, there were 2×10^8 CFU/ml of *P. aeruginosa* and 9×10^4 CFU/ml of *S. aureus* (Fig. 4A). Thus, in more prolonged culture in ASM, *P. aeruginosa* can outcompete an established population of *S. aureus*. Glucose in ASM continued to have a beneficial effect on the growth of *S. aureus* (ANOVA, $p < 0.001$, $n = 3$), and on the early recovery, but not long-term growth, of *P. aeruginosa* in this model.

3.7. *Stable co-culture of S. aureus and P. aeruginosa can be established after 24 h when grown in a 50:50 mix of ASM and supernatant*

To establish a *S. aureus* and *P. aeruginosa* co-culture model where both respiratory pathogens exist in balance, ASM was mixed 50:50 with pre-conditioned ASM with and without 4 mM glucose. Similar amounts of *S. aureus* and *P. aeruginosa* were added simultaneously

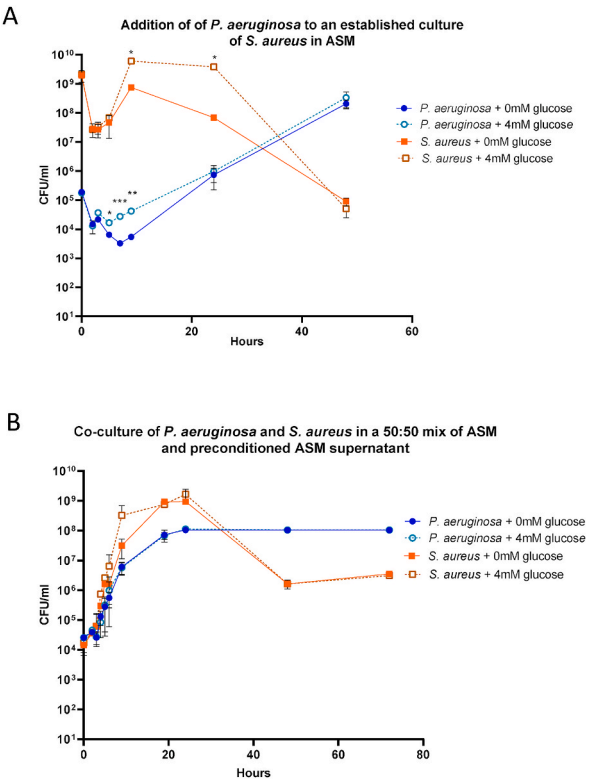

Fig. 4. Co-culture of *P. aeruginosa* and *S. aureus* in an established *S. aureus* culture and in 50:50 pre-conditioned ASM supernatant:ASM. A. 2×10^5 CFU/ml *P. aeruginosa* were added into established cultures of *S. aureus* grown with or without 4 mM glucose and with concentration of 2×10^9 CFU/ml. Growth of pathogens was observed over 48 h. B. Equal amounts of *S. aureus* and *P. aeruginosa* were added to 50:50 pre-conditioned ASM supernatant:ASM. Additional glucose was added at the stage of inoculation. Growth of pathogens was observed over 72 h. Data presented as mean ± SD. Effect of glucose was compared using mixed effects two-way ANOVA with Geisser-Greenhouse correction; *$p < 0.05$, **$p < 0.01$, ***: $p < 0.001$.

to the solution and both exhibited immediate growth, with *S. aureus* reaching 1×10^9 CFU/ml after 24 h of incubation, while *P. aeruginosa* reached 1×10^8 CFU/ml after 24 h of incubation (Fig. 4B). While *P. aeruginosa* remained stable in the culture at 5×10^8 CFU/ml, S. *aureus* decreased to 2×10^6 CFU/ml but remained at this level from 48 to 72 h (Fig. 4B). The presence of glucose in the co-culture did not significantly affect the growth of either *S. aureus* or *P. aeruginosa.* Thus, we demonstrated that we could establish a stable co-culture of *S. aureus* and *P. aeruginosa* over 72 h.

3.8. The growth of P. Aeruginosa in S. aureus pre-conditioned ASM is dependent on a heat labile factor

P. aeruginosa has been shown to utilise lactate produced by *S. aureus* in the lungs [25,28]. Lactate was present in ASM plus 4 mM glucose cultured with *S. aureus* in the first 2 h, but was quickly utilised in the subsequent 2 h and was undetectable by 5 h. At 6-h both glucose and lactate in the media could not be detected (Fig. 5A). Thus, we propose that in *S. aureus* conditioned medium, factors other than lactate promoted the growth of *P. aeruginosa*.

P. aeruginosa did not grow in pre-conditioned ASM that had been heated to 121 °C. Viable CFU decreased from 2×10^5 to 2×10^3 CFU/ml within the first 3 h of incubation and remained low (Fig. 5B). The effect on *P. aeruginosa* was independent of whether the pre-conditioned ASM contained glucose or not.

4. Discussion

In this study, we have demonstrated the potential to use ASM to study the effects of altered glucose concentration in the airway on the growth of common respiratory bacteria. While the addition of glucose did not affect the growth of *S. aureus* or *P. aeruginosa* in MH it promoted growth when added to ASM. It is common practice to use nutrient-rich media to explore the growth and interaction of these pathogens [23–27]. However, we propose that ASM better represents the nutrient status of ASL. In this environment, our data support previous findings that changes in glucose concentration promote the growth of *S. aureus* and *P. aeruginosa* [16,21,22,35,36]. We believe that our model for the stable co-culture of *S. aureus* and *P. aeruginosa* provides an *in vitro* model that better resembles *in vivo* CF scenario and allows exploration of changes to nutrients in this environment with high reproducibility.

Our data indicated that the strain of *S. aureus* we used better adapted to the low nutrient ASM that *P. aeruginosa* as it did not have

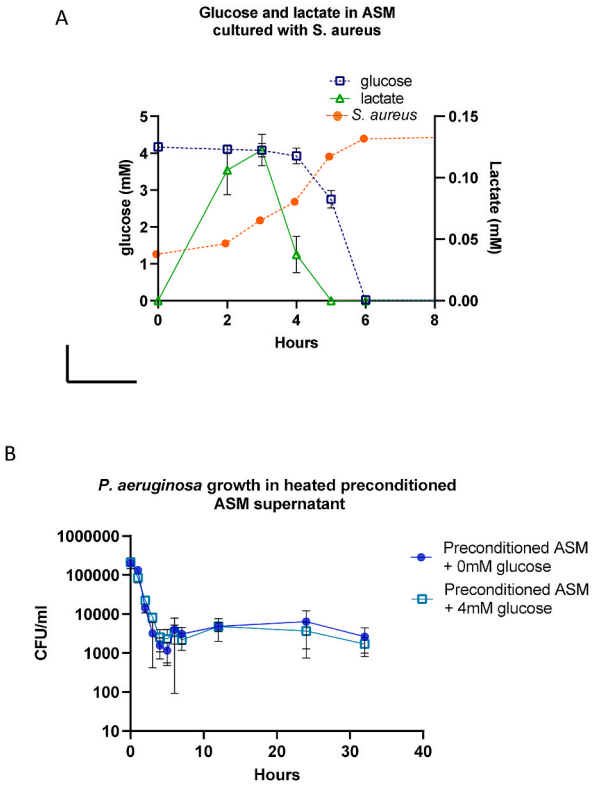

Fig. 5. Heat labile factors promote the growth of *P. aeruginosa* in pre-conditioned ASM supernatant. A. Lactate and glucose concentration during *S. aureus* culture in ASM over 24 h. The graph includes the data shown in Fig. 2A which has been overlaid for comparison. Presented as mean ± SD mM. Correlation between lactate and glucose concentration was calculated using Pearson correlation coefficient. B. *P. aeruginosa* was added to pre-conditioned ASM supernatant that had been heated to 121 °C. Growth was observed over 32 h. Presented as mean ± SD. Effect of glucose was compared using repeated measures two-way ANOVA with Geisser-Greenhouse correction.

the same lag in growth when inoculated into ASM. Both *P. aeruginosa* and *S. aureus* utilised glucose for growth in monoculture in the ASM. However, the role of glucose was less clear in co-culture. Studies have shown that in presence of *S. aureus, P. aeruginosa* downregulates genes responsible for sugar and carbohydrate metabolism [25]. This could explain why glucose did not induce changes to the growth of *P. aeruginosa* in co-culture, although it is possible that metabolic down-regulation could affect *P. aeruginosa* luminescent output, thus growth in this scenario could be underestimated. It was also reported that *P. aeruginosa* drives *S. aureus* from aerobic respiration towards fermentation pathways, in order to utilise the lactate produced by fermentation of glucose and *P. aeruginosa* increases the expression of lactate transporters, which allows it to utilise lactate as a preferential source of carbon [25, 28]. However, whilst our data indicate that in ASM with glucose, *S. aureus* did produce lactate, this was rapidly depleted by 6 h. It was therefore absent in the pre-conditioned ASM supernatant and likely absent in the established cultures of *S. aureus,* both of which rapidly promoted *P. aeruginosa* growth. Thus, our data indicate that factors other than glucose and lactate promoted the growth of *P. aeruginosa* in *S. aureus* pre-conditioned ASM. Nevertheless, we recognise that there are limitations to these experiments when comparing to the *in vivo* scenario [28]. ASM contained a finite supply of glucose, limiting lactate production. *In vivo,* with respiratory disease and hyperglycaemia, there is the potential for continuous glucose leak into the ASL for bacterial fermentation and lactate could enter the ASL from other cellular sources [37]. This work provides a starting point to further explore these interactions.

P. aeruginosa thrived in the environment pre-conditioned by *S. aureus.* Comprehensive evidence exists demonstrating that *S. aureus* and *P. aeruginosa* can modify each other's behaviour, pathogenicity and metabolism [38–42]. The majority of the studies, however, focus on the effects of *P. aeruginosa* on competitors including *S. aureus* [28,38–40,42,43]. *P. aeruginosa* can sense other microbial peptidoglycans and enhance the production of antimicrobial agents to suppress competition and increase its own virulence [44]. Thus, it is possible that peptidoglycans in the supernatant could modify *P. aeruginosa* growth. Relatively little is known about the effects of *S. aureus* on *P. aeruginosa.* In a human skin model of co-infection, *S. aureus* acts as a pioneer, priming the environment and promoting the attachment of *P. aeruginosa* to the keratinocytes [45]. Our data indicate that the *S. aureus* associated factor(s) in ASM that promoted *P. aeruginosa* were heat-labile. Peptidoglycans are likely to survive heat treatment. Therefore, it is currently difficult to speculate on the nature of these changes using existing literature, as previous transcriptomic and proteomic analysis demonstrated extreme metabolic plasticity of *P. aeruginosa*, and the ability to shift metabolism to adjust to the varying carbon sources depending on the environment [46]. A more thorough transcriptomic/proteomic study is now required to understand the interactions between *S. aureus* and *P. aeruginosa* in ASM and how *S. aureus* conditioned ASM promotes more rapid growth of *P. aeruginosa.*

Finally, mixing ASM with pre-conditioned ASM 1:1 allowed us to establish a relatively stable co-culture of *S. aureus and P. aeruginosa*. This now permits further exploration of the interaction between these organisms and others (e.g. *Haemophilus influenzae*), including clinical strains, factors that change in the ASL, and responses to antimicrobial agents [46].

In summary, we adapted an artificial sputum medium to create an *in vitro* model, that mimics the nutrient depleted *in vivo* lung environment to explore the effect of glucose on the growth and co-culture of two common respiratory pathogens. In this environment, glucose promoted the growth of *S. aureus* and *P. aeruginosa*, but other heat labile factors present in *S. aureus* conditioned medium also promoted the growth of *P. aeruginosa*. We suggest that our model of stable co-culture in ASM could be extrapolated to include clinical strains and other bacteria to better understand the growth and interaction of common lung pathogens in disease scenarios such as CFRD.

Data availability statement

Data is included in the article and supplementary material.

CRediT authorship contribution statement

Stanislavs Vasiljevs: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Arya Gupta:** Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. **Deborah Baines:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by an MRC Doctoral Training Partnership grant to S. Vasiljevs.

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