BICARBONATE INHIBITS THE GROWTH AND BIOFILM FORMATION OF PATHOGENS RELEVANT TO CYSTIC FIBROSIS

Dissertation Booklet

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1. Introduction

1.1. Cystic fibrosis lungs

Cystic fibrosis (CF) is a life-limiting genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1]. This gene encodes the CFTR protein, an epithelial anion channel found throughout the body, especially in the respiratory and gastrointestinal tracts. It primarily transports chloride (Cl^-) and bicarbonate (HCO_3^-) ions [2-4] and regulates sodium ion (Na^+) absorption via the epithelial sodium channels (ENaCs) [5]. These functions are very important for fluid balance across the epithelial cell layers. Therefore, mutations in the CFTR gene result in protein dysfunction, disrupting transepithelial electrolyte and water movements in many organs. Among the most severe complications, chronic lung disease and pulmonary exacerbation due to bacterial infection are the leading cause of death in CF.

In the respiratory tract, CFTR dysfunction causes a decrease in Cl^- and HCO_3^- secretion. This aberrant secretion alters the volume and composition of the airway surface liquid (ASL), resulting in a dehydrated and acidic mucus [6]. This mucus is persistent and very difficult to be removed by mucociliary clearance (MCC). Therefore, it accumulates and obstructs small airways. Inhaled bacteria trapped in this mucus cannot be adequately removed. These bacteria can also grow by consuming nutrients from the mucus. Body temperature and the slightly acidic pH also provide an optimal environment for bacterial growth and colonization [7]. In addition, acidic airway pH compromises the antimicrobial peptide activities [8]. Consequently, these factors lead to chronic airway infections, inflammation, and pulmonary failure [9, 10].

The most common bacteria found in the CF mucus are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Burkholderia cepacia* [11, 12]. These bacteria can form biofilms that persistently induce inflammation and worsen lung function. Biofilms provide structural stability and shield the bacteria, making them more resistant against host defense mechanisms and antibiotics. Aggressive antibiotic treatments are required. However, this could also aggravate antibiotic resistance.

1.2. Role of bicarbonate

Recent evidence points out that HCO_3^- may be the key for CF since HCO_3^- plays a pivotal role in airway physiology. Not only function as a CO_2 carrier for the gas exchange, HCO_3^- also involves the airway pH buffering system [13]. HCO_3^- can neutralize H^+ preventing a sudden pH change and sustaining the pH at approximately 7.0 ± 0.1 [14-16]. HCO_3^- also engages in normal mucus secretion. Mucin molecules are the building block of mucus. They are packed in secretory granules masked by H^+ and Ca^{2+}. Many studies suggest that HCO_3^- operates mucus secretion by chelating H^+ and Ca^{2+}, allowing expansion of mucin molecules [17, 18]. Moreover, the correlation between HCO_3^-, airway pH, and innate immunity has been recently reported [10]. It has been shown that under acidic conditions, the bacterial killing capacity of AMPs is significantly decreased. However, when ASL was supplemented with $NaHCO_3$, its bacterial killing capacity was restored [8, 19].

Over the last decade, accumulated evidence has hinted that HCO_3^- has also owned antimicrobial properties. It has been shown that natural mineral water that contains HCO_3^- can mitigate respiratory and skin infections [20]. In food and agricultural industries, HCO_3^- is used as an adjunctive microbial disinfectant and food preservative [21, 22]. In dentistry, HCO_3^- is added to dental care products, such as toothpaste and mouthwash, to reduce microbial accumulation and biofilm formation [23]. These findings have supported the antimicrobial property of HCO_3^-. However, it is still unclear whether this effect is due to its capacity to increase the pH or the anion *per se* that has antimicrobial effects.

1.3. Artificial sputum medium

In CF microbiology, the reliability of using microbiological media has been cast doubt since the composition of the CF lung secretion is different from that of the traditional media. Accumulated data also support that CF bacteria behave distinctively in the lungs, suggesting the importance of the unique environment. These notions led to the development of alternative culture media that resemble the habitat in CF airways [24-27].

Artificial sputum medium (ASM) is a mucin-based medium resembling the CF airway environment. Its first record appeared in 1997 by Ghani and Soothil, as they intended to induce *P. aeruginosa* biofilm formation [24]. Since there has never been a

consensus for the core recipe, various versions and modifications have been proposed. Sriramulu and colleagues pioneered the ASM study by investigating the effect of each ASM component and proposing the most comprehensive formula, containing mucins, free DNA, egg yolk emulsion, proteins, and electrolytes, as the main constituents [25]. Their finding later became the main guideline for creating ASM [28].

In the last decade, ASM has been applied in a broad range of studies. Accumulated evidence has proven its suitability in CF microbiology. CF pathogens grown in ASM tend to show their typical characteristics, including specific gene expression, microcolony (biofilm) formation, metabolite utilization, surface motility, evolutional diversity, and, most importantly, rigorous antibiotic-resistant ability, similar to those pathogens found in the CF lung [24, 25, 27, 29-35]. ASM has reduced the complexity of CF microbiology making it easily accessible in a laboratory setting.

2. Objectives

Although the antimicrobial property of HCO_3^- has been previously suggested, its direct effect on bacteria remains largely unknown. Thus, we have designed a series of experiments to investigate the antimicrobial effect of HCO_3^- *per se*. The experiments were performed both in conventional microbiological media and ASM.

The main goals were:

1. To determine the most suitable preparation procedure and composition of ASM for these experiments.

2. To study the effects of HCO_3^- on the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

3. To investigate the effects of HCO_3^- on biofilm formation of *Pseudomonas aeruginosa*.

3. Methods

3.1. Growth conditions

To investigate the effect of $NaHCO_3$ on CF bacteria, we designed and created specific growth conditions for the bacteria, summarized in Table 1.

Table 1. Culture media and conditions

Media	Conditions	pH	NaCl (mM)	$NaHCO_3$ (mM)	Atmospheric conditions	Glucose	DNA	Test
BHI	(1) Control BHI	7.4	-	-	Ambient air	-	-	OD
	(2) 100 mM NaCl	7.4	100	-	Ambient air	-	-	
	(3) 100 mM $NaHCO_3$	8.5	-	100	5% CO_2	-	-	
	(4) 100 mM $NaHCO_3$	7.4	-	100	20% CO_2	-	-	
Bouillon	(1) Control bouillon	7.5	-	-	Ambient air	×	-	CVA
	(2) Glucose bouillon	7.5	-	-	Ambient air	✓	-	
	(3) 100 mM NaCl	7.5	100	-	Ambient air	✓	-	
	(4) 50 mM $NaHCO_3$	7.5	-	50	10% CO_2	✓	-	
	(5) 100 mM $NaHCO_3$	7.5	-	100	20% CO_2	✓	-	
ASM_1	(1) $NaHCO_3$-free	7.4	100	-	Ambient air	✓	✓	CFU & CVA
Regular	(2) $NaHCO_3$-free	8.0	100	-	Ambient air	✓	✓	
	(3) 25 mM $NaHCO_3$	7.4	75	25	5% CO_2	✓	✓	
	(4) 100 mM $NaHCO_3$	8.0	-	100	5% CO_2	✓	✓	
ASM_2	(1) $NaHCO_3$-free	7.4	100	-	Ambient air	✓	×	FC
DNA-free	(2) $NaHCO_3$-free	8.0	100	-	Ambient air	✓	×	
	(3) 25 mM $NaHCO_3$	7.4	75	25	5% CO_2	✓	×	
	(4) 100 mM $NaHCO_3$	8.0	-	100	5% CO_2	✓	×	

Ambient air = 0.04% CO_2

OD = optical density; CFU = colony-forming unit assay; CVA = crystal violet assay; FC = flow cytometry.

Brain-heart infusion (BHI) medium (Mast Group Ltd., Merseyside, UK) was prepared following the manufacturer's instructions and modified to four different conditions. Bouillon medium was prepared according to the following recipe: 0.3% meat extract, 0.2% yeast extract, 1% peptone, and 0.5% NaCl and adjusted to five conditions.

ASM recipe was carefully developed based on published literature (see supplement material). It was prepared with four different conditions. ASM was also divided into two versions, regular and DNA-free. DNA-free ASM was used for the flow cytometry to avoid misinterpretation due to nucleic acid staining.

3.2. Bacterial strains

Bacterial strains used in the experiments are summarized in Table 2.

Table 2. List of Bacteria

Bacteria	Strains	Spectro-photometry	Colony-forming unit assay (CFU)	Flow cytometry	Biofilm crystal violet assay
S. aureus	ATCC® 29213™	✓	✓	✓	
S. aureus	SA-113	✓	✓		
P. aeruginosa	ATCC® 27853™	✓	✓	✓	
P. aeruginosa	PA-17808	✓	✓		✓

SA-113 and PA-17808 are clinical isolates

3.3. Growth experiments

3.3.1. Spectrophotometry

Overnight culture of each bacterium was inoculated to each BHI condition to the density of 0.5 McFarland adjusted with a VITEK Densichek apparatus (Biomérieux, Marcy l' Étoile, France). Aliquots of each suspension were dispensed into 96-well plates in duplicate and incubated at 37˚C in ambient air (for $NaHCO_3$-free BHI) or in 5% or 20% CO_2 (for $NaHCO_3$-containing BHI) as designated.

The growth of bacteria was followed by measuring the optical density (OD) at 595 nm using a PR2100 microplate reader (Bio-Rad Laboratories, Hercules, Canada). The growth rates were determined by calculating the area under the curve (AUC).

3.3.2. Colony-forming unit assay

Overnight cultures of each bacterium were adjusted to 3.0 McFarland and inoculated at a 1:50 dilution into ASM. Aliquots of each suspension were dispensed into 96-well plates in triplicate and incubated at 37˚C in ambient air (ASM without $NaHCO_3$) or 5% CO_2 (ASM with $NaHCO_3$). After 6 or 17 hours of incubation, bacterial culture was serially diluted over a range of dilution factors from 10^{-1} to 10^{-9}. Aliquots of each dilution were plated onto simple agar plates, incubated at 37˚C overnight.

The colonies on each plate were counted by using ImageJ software (NIH, USA). CFU per milliliter (CFU/mL) was calculated with the following equation and converted to a logarithmic scale (log CFU/mL).

$$CFU/mL = \frac{(Number\ of\ counts\ on\ the\ plate)}{(0.01 \times Dilution\ factor)}$$

3.3.3. Flow cytometry experiment

Bacterial samples were prepared in the same way as the CFU assay. After incubation, bacterial culture was collected, washed with 1 mL 0.85% NaCl solution twice. Then each bacterial suspension was adjusted to 0.5 McFarland with 0.85% NaCl solution. In experiments with *P. aeruginosa*, EDTA (5 mM) was added to the saline solution. Bacterial suspensions were stained (1:200) with LIVE/DEAD BacLight Bacteria Viability Kit (L7007, Invitrogen, Waltham, MA, USA) and incubated at room temperature in the dark for 25 min before the measurement.

Flow cytometry was carried out using BD FACSCalibur system (Becton Dickinson, San Jose, CA, USA) equipped with a 635-nm red diode laser and a 15-mW 488-nm air-cooled argon solid-state laser. Data were acquired with BD CellQuest Pro software (Becton Dickinson, San Jose, CA, USA) and analyzed with Flowing software version 2.5.1 (Turku Centre for Biotechnology, Turku, Finland, released 4.11.2013).

3.4. Biofilm experiments

Overnight cultures of *P. aeruginosa* (PA-17808) were inoculated in bouillon and ASM and incubated at 37 °C in ambient air or different CO_2 concentrations depending on the designated conditions for 48 h. After incubation, bacterial cultures were washed with PBS and stained with 0.1% violet solution. OD (595 nm) was measured.

3.5. Statistical analysis

Data were analyzed using Statistica for Windows 7.0 (StatSoft) and GraphPad Prism version 8 (GraphPad Software, Inc., San Diego, CA, USA). The values were compared using ANOVA followed by an LSD *post hoc* comparison test. Changes were considered statistically significant at $p < 0.05$.

4. Results

4.1. Effects of bicarbonate on the growth of bacteria prevalent in CF

4.1.1. Bicarbonate inhibits the growth of *S. aureus* and *P. aeruginosa* in BHI

The growth rate of both *S. aureus* and *P. aeruginosa* was significantly reduced in BHI broth supplemented with 100 mM $NaHCO_3$ equilibrated with 20% CO_2 (pH 7.4) compared to $NaHCO_3$-free BHI at the same pH value (Figure 1). Since the supplementation of 100 mM $NaHCO_3$ increased ionic strength, we used BHI supplemented with 100 mM NaCl (pH 7.4) as a control. Interestingly, the growth rate of both species was not affected in the NaCl-containing BHI, suggesting that the inhibitory effect of $NaHCO_3$ was not due to increased osmolality or ionic strength.

However, the growth inhibition was also observed in the alkaline BHI (pH 8.5) supplemented with 100 mM $NaHCO_3$ equilibrated with 5% CO_2, prompting the query if the alkalinity of $NaHCO_3$ could affect the bacterial growth. Therefore, $NaHCO_3$-free BHI (pH 8.5) was also tested. We found that the growth capacity was not affected in $NaHCO_3$-free BHI (pH 8.5). Additionally, the inhibitory effect in $NaHCO_3$-containing BHI (pH 8.5) was similar to that observed in $NaHCO_3$-containing BHI pH 7.4, indicating that the inhibitory effect was not due to alkaline pH.

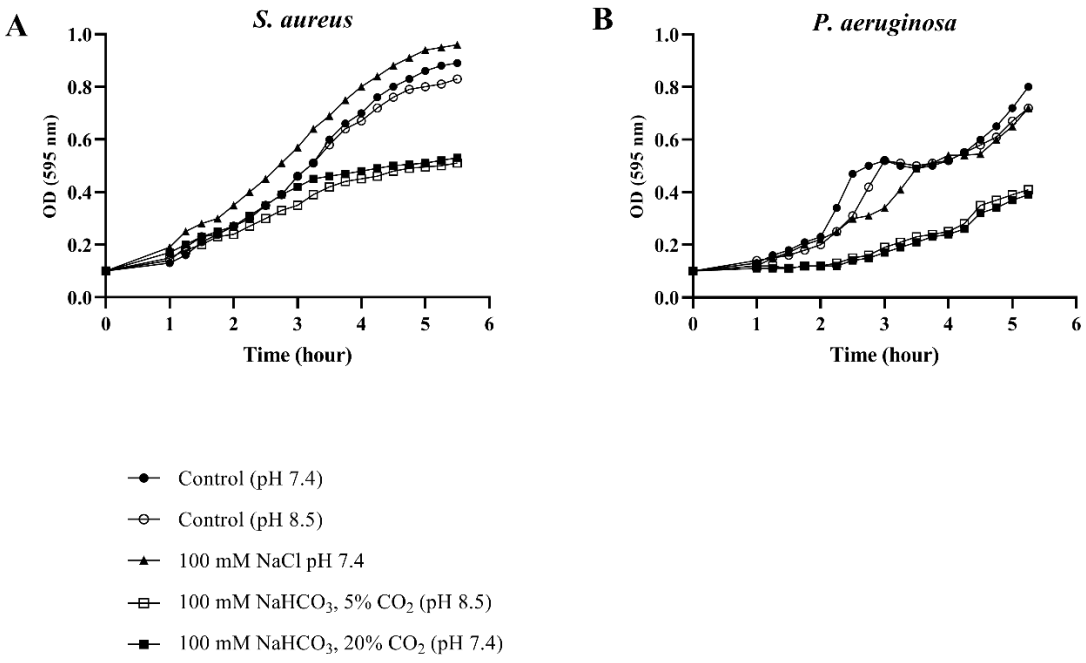

Figure 1. Bacterial growth rate in BHI medium with different conditions.
Growth of *S. aureus* **(A)** and *P. aeruginosa* **(B)** in BHI medium with different conditions, measured by the spectrophotometry technique.

The area under the curve (AUC) was calculated to compare the growth rates of bacteria in each condition quantitatively. The $NaHCO_3$-enriched medium resulted in approximately 25 to 50% AUC reduction for both species compared to the medium without $NaHCO_3$ (Table 3).

Table 3. Calculated AUC values based on growth curves in Figure 1.

Bacterium	BHI (pH 7.4)	BHI (pH 8.5)	100 mM NaCl (pH 7.4)	100 mM $NaHCO_3$ (pH 7.4)	100 mM $NaHCO_3$ (pH 8.5)
S. aureus	2.48	2.41	2.93	1.92	1.76
P. aeruginosa	2.05	1.92	1.82	1.01	1.06

4.1.2. Bicarbonate decreases the CFU of *S. aureus* and *P. aeruginosa* in ASM

Following 6 h incubation (Figure 2 A-D), the viable cell counts for both bacteria were significantly reduced in the ASM containing 100 mM $NaHCO_3$ (pH 8.0) compared

to $NaHCO_3$-free ASM (pH 8.0). In ASM supplemented with 25 mM $NaHCO_3$ (pH 7.4), only *P. aeruginosa* ATCC 27853 cell counts were significantly reduced (Figure 2 C).

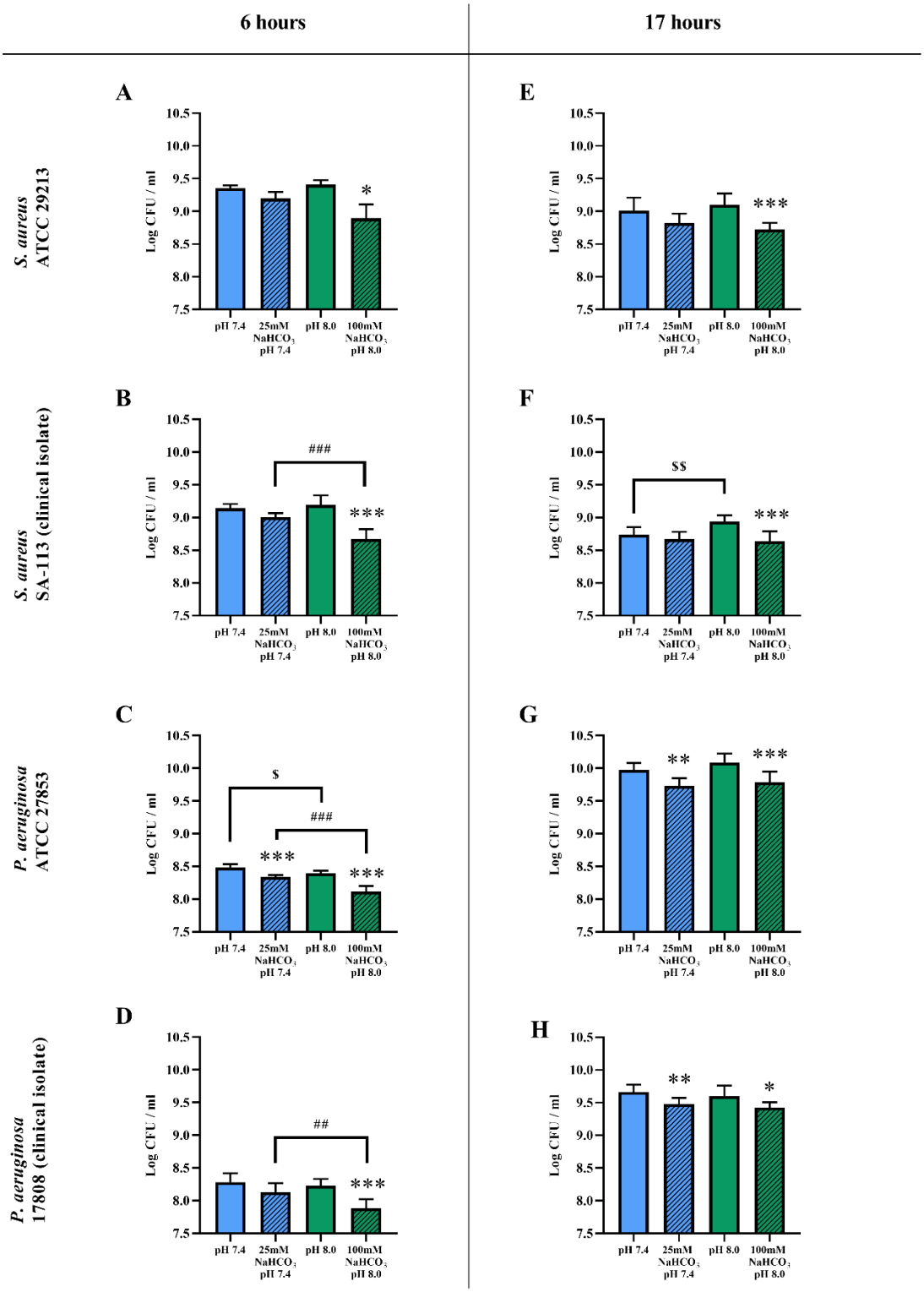

Figure 2. CFU assay of cystic fibrosis bacteria.

S. aureus ATCC 29213, *S. aureus* SA-113, *P. aeruginosa* ATCC 27853, and *P. aeruginosa* 17808 grown in different ASM conditions for 6 (**A-D**) and 17 h (**E-H**) in ambient air or 5% CO_2. Values are presented as mean ± SD.

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ when comparing between same-colored columns.
= $p < 0.01$, ### = $p < 0.001$ when comparing between shaded columns.
$ = $p < 0.05$, $$ = $p < 0.01$ when comparing between clear columns.

After a 17 h incubation (Figure 2 E-H), similar inhibitory effects of $NaHCO_3$ were observed. The viable cell counts for both bacteria were significantly decreased in the ASM containing 100 mM $NaHCO_3$ (pH 8.0). However, in ASM containing 25 mM $NaHCO_3$ (pH 7.4), only viable cell counts for *P. aeruginosa* (the ATCC strain and the clinical isolate) were significantly reduced.

Comparing the two $NaHCO_3$-containing ASM (25 *vs.* 100 mM $NaHCO_3$), the viable cell count reduction caused by 100 mM $NaHCO_3$ was significantly greater than that caused by 25 mM $NaHCO_3$ at 6 h incubation (with the single exception of the *S. aureus* ATCC strain) (Figure 2 B-D). These results suggest a concentration-dependent inhibitory effect of HCO_3^- on bacterial growth. However, no significance was detected in either species after the longer incubation (17 h) (Figure 2 E-H).

In the absence of $NaHCO_3$, the more alkaline (pH 8.0) medium did not decrease the *S. aureus* cell counts compared to the pH 7.4 (Figure 2 A, B&E). Interestingly, the counts were increased at pH 8.0 in the *S. aureus* clinical isolate following the 17 h incubation (Figure 2 F). On the other hand, the more alkaline pH slightly reduced the *P. aeruginosa* ATCC cell count after the 6 h incubation (Figure 2 C), but there was no significant difference following 17 h incubation (Figure 2 G). There was no significant difference in P. aeruginosa clinical isolate regardless of incubation time (Figure 2 D&H).

Taken together, our CFU data suggest that $NaHCO_3$ has a concentration-dependent inhibitory effect on bacterial growth, which is not due to changes in external pH.

4.1.3. Bicarbonate decreases the number of viable cells and increases membrane-damaged cells detected by the flow cytometry

We found a significant reduction in the percentage of SYTO9-positive cells for both bacteria in the ASM containing 100 mM $NaHCO_3$ (pH 8.0) compared to $NaHCO_3$-free ASM at the same pH (Figure 3 A&C). In contrast, the percentage of PI-positive cells was significantly increased in the ASM containing 100 mM $NaHCO_3$ for both species as well, when compared to $NaHCO_3$-free ASM at the same pH (pH 8.0) (Figure 3 B&D). Interestingly, in *P. aeruginosa*, a significant increase in the percentage of PI-positive cells in ASM containing 25 mM $NaHCO_3$ (pH 7.4) was also detected (Figure 3 D).

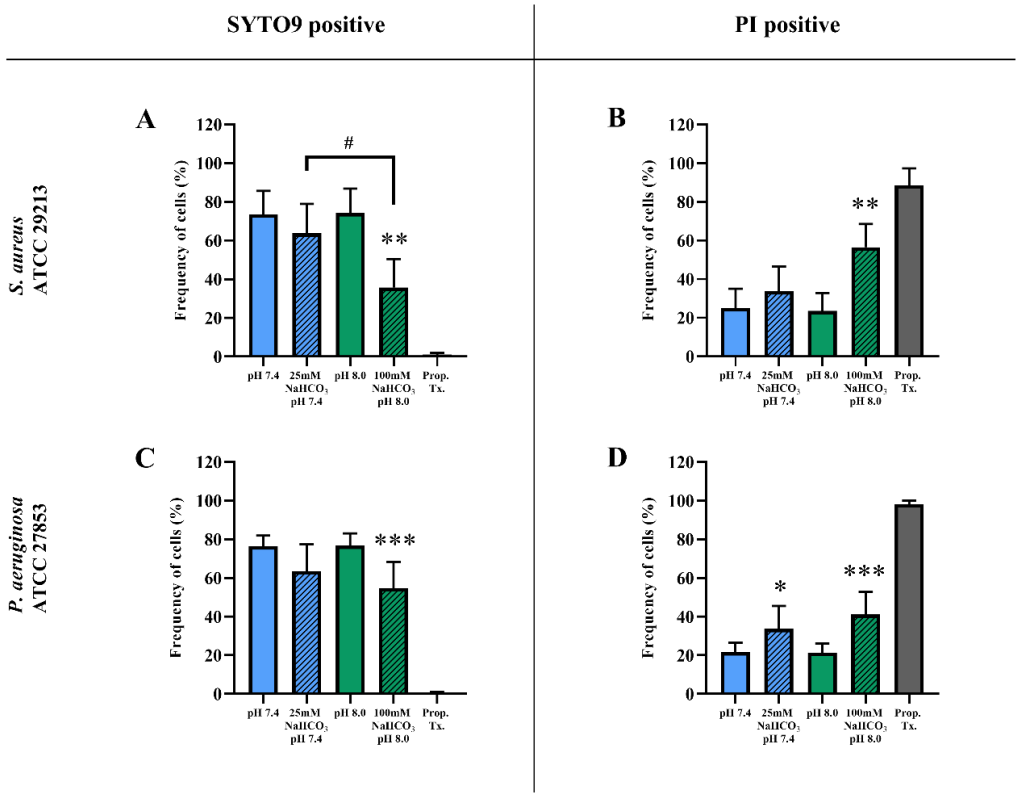

Figure 3. Percentage of SYTO9- and PI-positive signals.

Values are calculated from SYTO9- and PI-positive clusters of *S. aureus* ATCC 29213 (**A&B**) and *P. aeruginosa* ATCC 27853 (**C&D**) grown in different ASM after 17 h of incubation, and presented as a mean ± SD.

** = $p < 0.01$, *** = $p < 0.001$ when comparing between same-colored columns.

= $p < 0.05$ when comparing between shaded columns.

In addition, we observed a concentration-dependent decrease in the percentage of SYTO9 positive *S. aureus* cells when compared the effects of 25 mM $NaHCO_3$ (pH 7.4) and 100 mM $NaHCO_3$ (pH 8.0) (Figure 3 A). In fact, this concentration-dependent pattern could be detected for the percentage of SYTO9 positive *P. aeruginosa* cells and the percentage of PI-positive of both bacteria species. However, differences did not reach the level of statistical significance. Furthermore, the ratios of SYTO9 to PI-positive cells of both bacteria species remain unchanged in $NaHCO_3$-free ASM regardless of pH (pH 7.4 vs. 8.0) (Figure 4). These results suggest that the effects of $NaHCO_3$ were not due to the alkalinization of the medium.

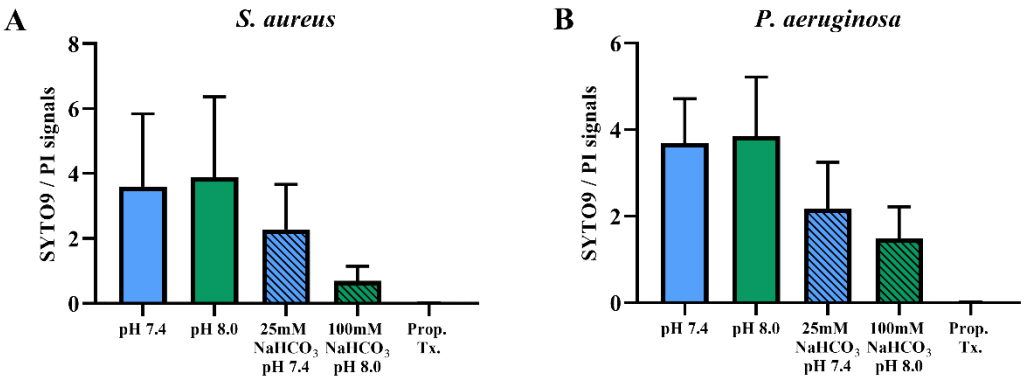

Figure 4. Ratios of SYTO9- to PI-positive signals.

S. aureus ATCC 29213 (**A**) and *P. aeruginosa* ATCC 27853 (**B**) in different ASM media. Values are presented as means ± SD.

4.2. Effects of bicarbonate on *P. aeruginosa* biofilm formation

4.2.1. Bicarbonate inhibits biofilm formation in conventional medium

Robust biofilm formation was observed in the $NaHCO_3$-free bouillon following 48 h of incubation, whereas biofilm formation was almost completely inhibited in bouillon containing either 50 or 100 mM $NaHCO_3$ (Figure 5 A). Surprisingly, the NaCl-containing medium drastically increased biofilm formation compared to that observed in $NaHCO_3$-containing bouillon media, suggesting that the inhibition of biofilm formation was due to the effect of HCO_3^- *per se*.

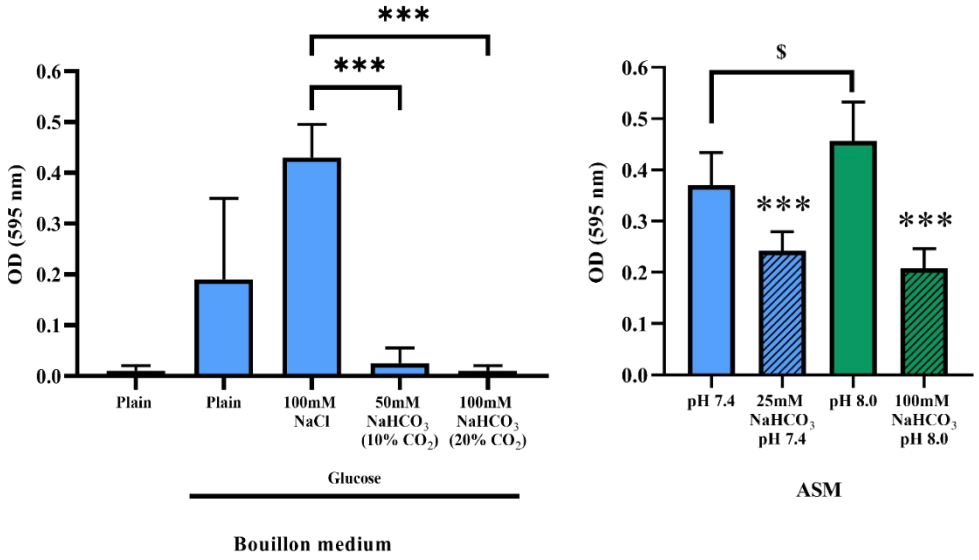

Figure 5. Biofilm formation by *P. aeruginosa* in bouillon media (Left) and ASM (Right). *** $p < 0.001$.

4.2.2. Bicarbonate inhibits biofilm formation in ASM

P. aeruginosa biofilm formation was detected in $NaHCO_3$-free ASM following 48 h incubation. In contrast, biofilm formation was significantly inhibited in NaHCO3-containing ASM (both 25 and 100 mM) (Figure 5 B). Interestingly, the alkaline $NaHCO_3$-free ASM (pH 8.0) increased biofilm formation. This evidence reinforces the assumption that the high pH does not inhibit biofilm formation.

5. Conclusions

In this study, we successfully developed a suitable preparation procedure to prepare a unique medium (ASM) that resembles the CF airway environment to study the effects of HCO_3^- *per se* on CF bacteria.

HCO_3^- inhibits the growth of *S. aureus* and *P. aeruginosa*, the most prevalent CF bacteria. HCO_3^- also suppresses *P. aeruginosa* biofilm formation. Importantly, we detected these inhibitory effects in both conventional medium and ASM. The composition of the latter medium resembles the CF airway mucus, suggesting that the inhibitory effects of HCO_3^- might also exist in CF lung. We demonstrated that these effects are independent of changes in pH and osmolality, suggesting that the inhibitory effects are

merely induced by HCO_3^- *per se*. We also showed that HCO_3^- effects were concentration-dependent.

Taken together, we demonstrated that HCO_3^- has antimicrobial effects. Furthermore, HCO_3^- restores airway pH, mucus secretion, and bacterial killing capacity. Since recent evidence has also suggested that HCO_3^- is safe on bronchial epithelial cells, inhalation of aerosolized HCO_3^- could be a potential symptomatic therapy for CF lung disease and other airway diseases (e.g., COPD) associated with mucus accumulation and bacterial infection.

6. List of Own Publications

Original publications within the topic of the Ph.D. thesis:

1. Dobay, O., Laub, K., Stercz, B., Kéri, A., Balázs, B., Tóthpál, A., Kardos, S., **Jaikumpun, P.**, Ruksakiet, K., Quinton, P.M., and Zsembery, Á. (2018). Bicarbonate Inhibits Bacterial Growth and Biofilm Formation of Prevalent Cystic Fibrosis Pathogens. *Front Microbiol.* 9, 2245.
   Journal Article/Article (Journal Article)/Scientific
   SJR Scopus - Microbiology (medical): Q1
   IF: 4.259

2. Gróf, I., Bocsik, A., Harazin, A., Santa-Maria, A.R., Vizsnyiczai, G., Barna, L., Kiss, L., Fűr, G., Rakonczay, Z., Jr., Ambrus, R., Szabó-Révész, P., Gosselet, F., **Jaikumpun, P.**, Szabó, H., Zsembery, Á., and Deli, M.A. (2020). The Effect of Sodium Bicarbonate, a Beneficial Adjuvant Molecule in Cystic Fibrosis, on Bronchial Epithelial Cells Expressing a Wild-Type or Mutant CFTR Channel. *Int J Mol Sci.* 21, 4024.
   Journal Article/Article (Journal Article)/Scientific
   IF: 5.923

3. **Jaikumpun, P.**, Ruksakiet, K., Stercz, B., Pállinger, É., Steward, M., Lohinai, Z., Dobay, O., and Zsembery, Á. (2020). Antibacterial Effects of Bicarbonate in Media Modified to Mimic Cystic Fibrosis Sputum. *Int J Mol Sci.* 21.
   Journal Article/Article (Journal Article)/Scientific

IF: 5.923

4. Ruksakiet, K., Stercz, B., Tóth, G., **Jaikumpun, P.**, Gróf, I., Tengölics, R., Lohinai, Z.M., Horváth, P., Deli, M.A., Steward, M.C., Dobay, O., and Zsembery, Á. (2021). Bicarbonate Evokes Reciprocal Changes in Intracellular Cyclic di-GMP and Cyclic AMP Levels in *Pseudomonas aeruginosa*. *Biology.* 10, 519.
   Journal Article/Article (Journal Article)/Scientific
   SJR Scopus - Agricultural and Biological Sciences (miscellaneous): D1
   IF: 5.079
5. Budai-Szűcs, M., Berkó, S., Kovács, A., **Jaikumpun, P.**, Ambrus, R., Halász, A., Szabó-Révész, P., Csányi, E., and Zsembery, Á. (2021). Rheological effects of hypertonic saline and sodium bicarbonate solutions on cystic fibrosis sputum *in vitro*. BMC Pulm Med. 21, 225.
   IF: 4.1

Supplement material. Artificial sputum medium recipe developed from published literature.

Author	*Ghani & Soothill*	*Sriramulu et al.*	*Palmer et al.*	*Fung et al.*	*Kirchner et al.*	*Yeung et al.*	*Wright et al.*	*Behrends et al.*	*Quinn et al.*	*Comstock et al.*	*Davies et al.*	*Jaikumpun et al.*	Reference
Year	1997	2005	2007	2010	2012	2012	2013	2013	2015	2017	2017	**2020**	
mucin	0.5%	0.5%	-	1%	0.5%	1%	0.5%	0.5%	2%	2%	0.5%	**2%**	[33, 36]
DNA	4 g/L	4 g/L	-	1.4 g/L	4 g/L	1.4 g/L	4 g/L	4 g/L	1.4 g/L	1.4 g/L	4 g/L	**1.4 g/L**	[27, 30, 33, 36]
NaCl	90 mM Na^+ 30 mM K^+ 80 mM Cl^-	85 mM	50 mM	85 mM	85 mM	50 mM	85 mM	85 mM	85 mM	100 mM	85 mM	**100 mM**	[36]
KCl		30 mM	15 mM	30 mM	30 mM	15 mM	30 mM	30 mM	30 mM	30 mM	30 mM	**30 mM**	[28, 29, 33, 36]
Ferritin		-	-	-	-	-	-	-	3 µL/mL	3 µL/mL	-	**3 µL/mL**	[33, 36-39]
DTPA	5.9 mg/L	5.9 µg/mL	-	5.9 µg/mL	5.9 µg/mL	-	5.9 µg/mL	5.9 µg/mL	-	-	5.9 µg/mL	-	[33, 36]
Egg yolk	5 mL/L lecithin	5 µL/mL	-	5 µL/mL	5 µL/mL	-	5 µL/mL	5 µL/mL	0.01%	5 µL/mL	5 µL/mL	**5 µL/mL**	[28, 29, 36]
Essential amino acids		5 g/L	19 mM	5 g/L	2.5 g/L	1 g/L	2.5 g/L	5 g/L	7.225x	7.225x	2.5 g/L	**5 g/L**	[28]
Non-essential amino acids									14.45x	7.225x			
Glucose	-	-	3 mM	-	-	-	-	-	-	-	-	**11 mM**	[40]
Buffer	Tris	Tris	PO_4^{3-} & MOPS	Tris	Tris	PO_4^{3-}	Tris	Tris	PO_4^{3-}		Tris	**HEPES & $NaHCO_3$**	-
Other modifications		-	9.3 mM L-lactate, 2.3 mM NH_4Cl	10 mg/mL BSA	-	2 mM $MgSO_4$, 10 µM $FeSO_4$	-	-	-	Indicators	-	**$NaHCO_3$**	[40]
Sterilization	-	Autoclave	-	Antibiotics	Filtration	70°C 24h	Filtration	Filtration	Autoclave	Autoclave	Filtration	**Multiple techniques**	-

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