Universidade do Minho
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UMinho | 2023

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Dissertação de Mestrado
Mestrado em Biotecnologia

Trabalho efetuado sob a orientação da
Doutora Paula Alexandra da Silva Jorge

janeiro de 2023

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Acknowledgements

The help and support of several people were crucial for the accomplishment of the dissertation, and therefore, I am grateful to all the people who contributed directly or indirectly in some way to the completion of my dissertation.

To my supervisor, Dr. Paula, for sympathy, availability, dedication, patience, comprehension, and coordination. For all the knowledge transmitted at a theoretical level as well as in terms of laboratory techniques. For all the support, critical thinking, suggestions, and clarification of all my questions during this work. Grateful for everything!

To the MOP laboratory group, for the good atmosphere, sympathy, availability, and clarification of all procedures and questions throughout the development of the laboratory work.

To my family and friends in general, for the moments of sharing and happiness, companionship, dedication, care, support, and exchange of experience that made me evolve personally and academically.

To my parents, words will never be enough to thank them. Thank you for helping me in everything, for giving me confidence, for believing in me and in my capacity to continue to accomplish my goals.

Finally, to Nelson, my great support, for making my days happier, thank you for your care, comprehension, attention, patience, knowledge sharing, and encouragement to overcome the difficulties that arose throughout this dissertation.

I'm grateful to everyone.

STATEMENT OF INTEGRITY

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Novas terapias para infecções pulmonares relacionadas com a DPOC por inspeção de interações bacterianas

Resumo

Anualmente, milhões de pessoas morrem devido à doença pulmonar obstrutiva crónica (DPOC). Esta doença incurável é marcada por exacerbações que são principalmente provocadas por bactérias formadoras de biofilmes, onde NTHi e a sua interação com outras bactérias aumentam a resistência aos agentes antimicrobianos. Portanto, entender o papel das bactérias e das suas interações na DPOC é crucial para o desenvolvimento de novos fármacos capazes de combater a resistência destas aos antibióticos.

O principal objetivo desta dissertação consistiu no estudo das condições ótimas de crescimento de biofilmes de *H. influenzae* e de *S. aureus* e das interações bacterianas que se estabelecem nesses biofilmes, bem como a avaliação da suscetibilidade das duas espécies a uma seleção de péptidos antimicrobianos (AMPs).

O impacto da renovação do meio de cultura em biofilmes de *H. influenzae* revelou inexistência de células cultiváveis quando este não foi renovado às 24 h, possivelmente devido a metabolitos tóxicos, ao esgotamento de nutrientes, ou à existência de células viáveis mas não cultiváveis. Os resultados demonstraram que as diferenças no crescimento de *H. influenzae* em diferentes marcas do meio de crescimento (Oxoid, Liofilchem e VWR) podem dever-se à qualidade do meio usado, dado que o meio mais caro (Oxoid) foi o que resultou em menos variabilidade, tanto em biofilmes de uma ou duas espécies. Portanto, foi decidido usar esta marca para os ensaios subsequentes. Os resultados da dinâmica populacional de biofilmes mistos de *H. influenzae* com diferentes concentrações iniciais de *S. aureus* sugerem que ambas as espécies podem estar a beneficiar o crescimento uma da outra. Do estudo da influência da colonização sequencial nos biofilmes, não se verificou a influência das espécies colonizadoras na formação dos biofilmes de outra espécie. Na avaliação do efeito dos exoprodutos produzidos por uma espécie na formação de biofilmes da outra espécie, não se observaram quaisquer efeitos, pelo que a concentração dos exoprodutos pode ter sido insuficiente para causar um impacto ou as células do biofilme podem não ser responsivas aos exoprodutos produzidos. Por fim, o AMP tachyplesin I demonstrou melhor ação bacteriostática e bactericida contra ambas as estirpes em estudo, revelando resultados promissores para trabalhos futuros.

Os resultados deste trabalho permitiram extrair informações úteis, nomeadamente condições ótimas de crescimento e tipo de interações estabelecidas *in vitro*, que ajudarão o grupo de pesquisa na busca de potenciais agentes antimicrobianos para combater os biofilmes formados por estas bactérias.

Palavras-chave: DPOC; Exacerbações; *Haemophilus influenzae*; Interações bacterianas; *Staphylococcus aureus*.

New therapies for COPD-related pulmonary infections unveiled by inspecting bacterial interactions

Abstract

Every year, millions of people die from chronic obstructive pulmonary disease (COPD). This incurable disease is marked by exacerbations that are mainly caused by biofilm-forming bacteria, where NTHi and its interaction with other bacteria increase resistance to antimicrobial agents. Therefore, understanding the role of bacteria and their interactions in COPD is crucial for the development of new drugs capable of combating their resistance to antibiotics.

The main objective of this dissertation was to study the optimal conditions for the growth of *H. influenzae* and *S. aureus* biofilms and the bacterial interactions that are established in these biofilms, as well as the evaluation of the susceptibility of the two species to a selection of antimicrobial peptides (AMPs).

The impact of renewing the culture medium on *H. influenzae* biofilms revealed the absence of culturable cells when the medium was not renewed after 24 h, possibly due to toxic metabolites, nutrient depletion, or the existence of viable but non-culturable cells. The results showed that the differences in *H. influenzae* growth in different brands of growth medium (Oxoid, Liofilchem, and VWR) could be due to the quality of the medium used, given that the most expensive medium (Oxoid) was the one that resulted in less variability, both in biofilms of one or two species. Therefore, it was decided to use this brand for subsequent assays. The population dynamics results of mixed *H. influenzae* biofilms with different initial concentrations of *S. aureus* suggest that both species could be benefiting each other's growth. From the study of the influence of sequential colonization on biofilms, the influence of colonizers species on the formation of biofilms of another species was not verified. In assessing the effect of exoproducts produced by one species on biofilm formation by another species, no effects were observed, so the concentration of exoproducts may have been insufficient to cause an impact, or the biofilm cells may not be responsive to the exoproducts produced. Finally, AMP tachyplesin I showed better bacteriostatic and bactericidal action against both strains under study, revealing promising results for future work.

The results of this work allowed extracting useful information, namely optimal growth conditions and type of interactions established *in vitro*, which will help the research group in the search for potential antimicrobial agents to combat the biofilms formed by these bacteria.

Keywords: Bacterial interactions; COPD; Exacerbations; *Haemophilus influenzae*; *Staphylococcus aureus*.

Graphical Abstract

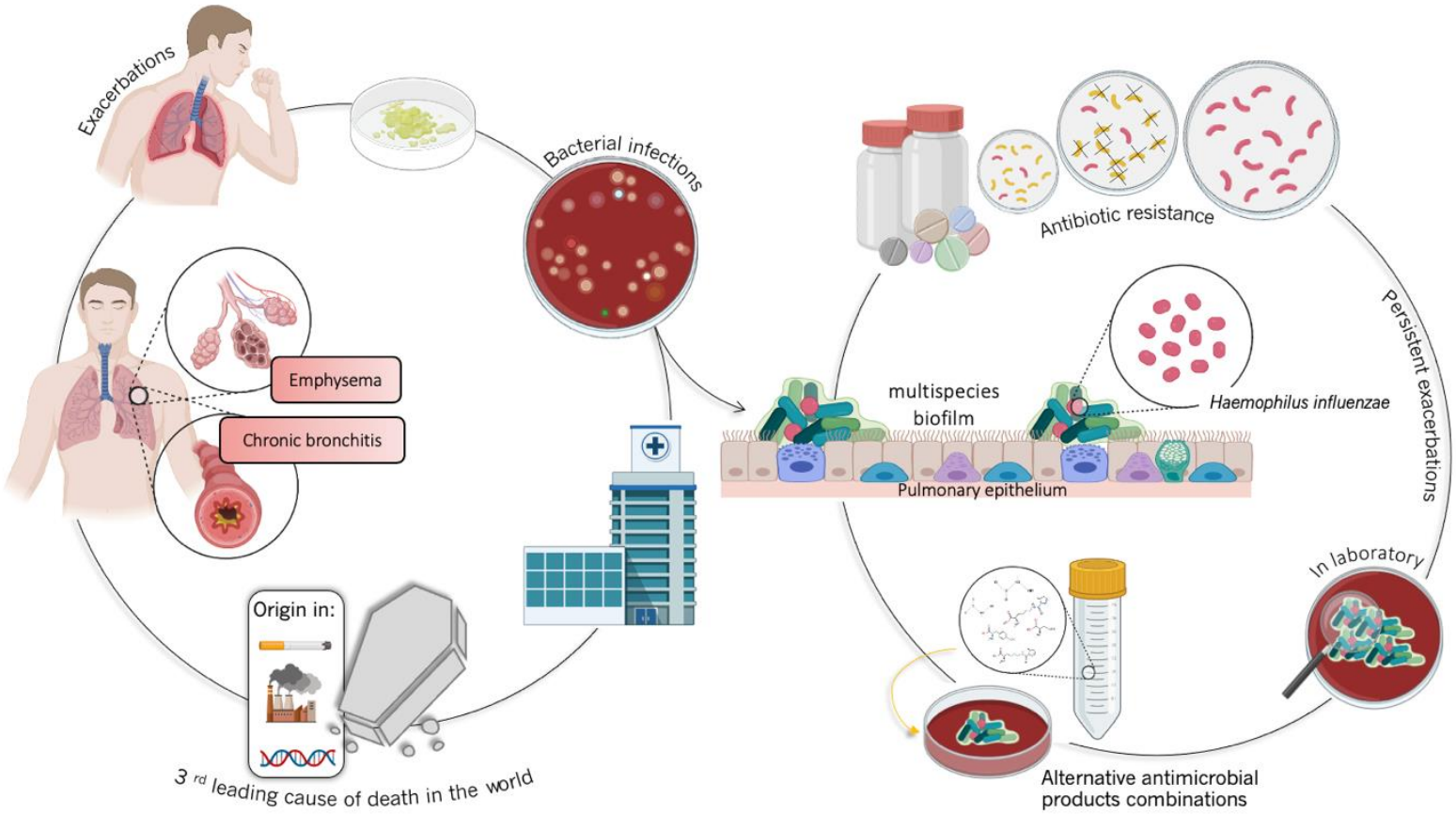

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Table of Contents

List of Abbreviations

AECOPD	Acute Exacerbations of Chronic Obstructive Pulmonary Disease
AMP	Antimicrobial Peptide
BHI	Brain Heart Infusion
BHIA	Brain Heart Infusion Agar
CFU	Colony Forming Units
COPD	Chronic Obstructive Pulmonary Disease
CV	Crystal Violet
DNA	Deoxyribonucleic acid
ECM	Extracellular Polymeric Matrix
eDNA	Extracellular Deoxyribonucleic acid
EPS	Extracellular Polymeric Substances
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
GOLD	Global Initiative for Chronic Obstructive Lung Disease
LOS	Lipooligosaccharide
MBC	Minimal Bactericidal Concentration
MHB	Mueller Hinton Broth
MH-F	Mueller Hinton broth supplemented with 5% lysed horse blood and 20 mg/L β-NAD
MIC	Minimal inhibitory Concentration
MSA	Mannitol Salt Agar
NAD	Nicotinamide Adenine Dinucleotide
OD	Optical Density
PBP	Penicillin Bind Protein
RNA	Ribonucleic acid
rpm	Rotations Per Minute
ROS	Reactive Oxygen Species
SABDs	Short-acting Bronchodilators
sBHI	Supplemented Brain Heart Infusion Broth
sBHIA	Supplemented Brain Heart Infusion Agar

SD	Standard Deviation
WHO	World Health Organization

List of Figures

List of Tables

Chapter 1. Introduction

This chapter presents the contextualization and motivation of this dissertation, namely explaining the severity of COPD, characterizing the pathology, revealing the main bacteria responsible for the critical periods of the disease, and not least, the problem in the treatment of these infections due to antibiotic resistance by these microorganisms. Furthermore, this chapter conveys the main objectives of this dissertation given this scenery and the structure of the document.

1.1 Context and Motivation

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide that mainly affects the respiratory system (GOLD, 2022). According to the World Health Organization (WHO), in 2019, 3.23 million people died due to COPD, with almost 90% of these deaths reported in LMICs (WHO, 2021). COPD is a common, preventable, and progressive debilitating disease that although treatable, it has no cure and its appearance is mainly related to smoking habits, genetic factors, and pollution (Awokola et al., 2022; Jiang et al., 2016; Weinberger et al., 2019; WHO, 2021). This disease has a high socio-economic burden and includes two pathologies, namely emphysema, demarcated by the destruction of the lung parenchyma, and chronic bronchitis, characterized by mucus hypersecretion, resulting in chronic productive cough (GOLD, 2022; Leung et al., 2017; Vogelmeier et al., 2020).

COPD is characterized by frequent exacerbations that are denoted by a sudden decline in lung function and the consequent worsening of symptoms (GOLD, 2022). These exacerbations are caused by pathogenic bronchial colonization, of which 50% are related to bacterial infections, often resulting in poor quality of life, increased hospitalizations, and higher mortality rates (Saxena et al., 2016; Vogelmeier et al., 2020). These bacterial infections are the result of bacterial adhesion to the pulmonary epithelium and the consequent formation of biofilms (Short et al., 2021). Biofilms are aggregates of bacteria in a self-produced polymeric matrix that normally include different microbial species, which interact with each other benefiting or harming one another (Melton & Anderson, 2019; Welp & Bomberger, 2020). Specifically, in COPD exacerbations, non-typable *Haemophilus influenzae* (NTHi) is the most prevalent isolated species, followed by *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (Leung et al., 2017; Short et al., 2021; Su et al., 2018).

In recent times, there has been a global increasing development of antibiotic resistance on the part of bacteria (Jorge et al., 2019). This resistance compromises the effectiveness of infection treatments

and leads to the selection of resistant subpopulations that can make COPD exacerbations even more persistent and severe (Welp & Bomberger, 2020). It is, therefore, crucial to fully understand the interactions between the bacteria present in COPD-related infections in order to try to find new therapies adapted to this polymicrobial reality. Thus, the focus of this work was the study of *H. influenzae* and *S. aureus* biofilm interactions. *H. influenzae* was selected given its high relevance in COPD related infections, and *S. aureus* was chosen because it is the second least studied of the four prevalent species (*M. catarrhalis*, *S. pneumoniae*, and *P. aeruginosa*). In fact, up until February 2020, only nine articles were published relating *S. aureus* with *H. influenzae* in COPD, most of which only analyzed the co-occurrence of the two species in patients and none of studied them in the context of a biofilm (Amaro, 2022).

1.2 Main Objectives

The main objective of this dissertation is to understand and characterize the biogeography of the polymicrobial consortia that often establishes in the lungs of patients with COPD, always focusing on NTHi. In this sense, this work was focused on the investigation of interactions of two species, namely *H. influenzae* and *S. aureus*, in biofilms, to try and identify the social relationships that they establish with each other. The rationale was to obtain information on the influence of growth conditions, specifically the brand of media used on *H. influenzae* and *S. aureus* biofilm formation, as well as the population dynamics between the two species with different initial concentrations. Sequential colonization was also investigated to understand whether the introduction of a species at a later stage induces changes to the biofilm and, moreover, if there is any benefit or damage to the total community. Finally, the effect of extracellular products that may eventually bring some benefit or harm to the biofilm community was also investigated, as well as the susceptibility of the bacteria to potential therapeutic agents, more specifically antimicrobial peptides (AMPs). This thesis was performed within the scope of an ESCMID Research Project, hence the information here acquired will later assist in the selection and testing of antimicrobial products for the eradication of these multispecies aggregates, with the ultimate goal of developing and evaluating new therapeutic strategies to treat COPD-related infections.

1.3 General Outline of the Thesis

The structure of the thesis is composed by five chapters. Chapter 1 addresses the context, scope, main objectives, and organization of this thesis. Chapter 2 presents the state of the art encompassing theoretical knowledge about COPD, the relevance of NTHi in COPD, and the role of biofilms in the development of the disease. In addition, it addresses the increasing resistance to antibiotics by bacteria

and the role of antimicrobial peptides to treat COPD infections. Chapter 3 describes the procedures, the strains, the growth media, and the main material used to out the experiments. Chapter 4 reveals the results obtained during the experiments performed and their discussion. Finally, Chapter 5 contains the main conclusions and suggested future work.

Chapter 2. State of the Art

Chapter 2 presents the definition of COPD, describes the two pathologies associated with this disease, its origin, and the acute periods of the disease. Furthermore, it gives theorical knowledge about the bacteria involved in the COPD related infections and the strategical mechanisms that allow them to survive in the hosts. This section also addresses the impact of antimicrobial resistance in COPD exacerbation treatment as well as the promise laying in antimicrobial peptides to treat them.

2.1 Chronic Obstructive Pulmonary Disease (COPD)

2.1.1 Definition

Chronic Obstructive Pulmonary Disease (COPD) is a common progressive disease that is currently considered the most prevalent chronic respiratory disease worldwide (Frazer, 2020; Hatipoglu, 2018). It is one of the leading causes of death in the world, presenting high rates of mortality and morbidity, being an economically expensive public health problem (Brody et al., 2020; Frazer, 2020; GOLD, 2021). In this disease, structural (central airways, peripheral airways, lung parenchyma, and pulmonary vasculature) and functional (gas exchange, inflation, and airflow) changes occur in the lungs, being mainly characterized by airway obstruction and persistent airflow limitation (Davidson & Bai, 2005; Papandrinopoulou et al., 2012). Generally, these limitations are caused by excessive mucus production, thickening of airway walls resulting from edema or muscle hypertrophy, and structural changes in lung tissue, such as loss of lung elasticity and tissue destruction (Frazer, 2020; Papandrinopoulou et al., 2012; Vogelmeier et al., 2017).

COPD includes two disorders, namely chronic bronchitis and emphysema. Chronic bronchitis is caused by changes in the mucus-secreting system (Weinberger et al., 2019). In this case, there is an increase in mucus-secreting glands and goblet cells responsible for bronchial secretions. This increase induces thickening of the airway walls and excessive production of mucus causing blockage of the lumen (Papandrinopoulou et al., 2012). In addition, the bronchial walls show cell infiltration and fibrosis resulting from the inflammatory process (Weinberger et al., 2019). In turn, emphysema is a pathology characterized by destruction of the parenchyma (alveolar walls), enlargement of air spaces distal to the terminal bronchiole, loss of elastin, and abnormalities in the formation of the lung elastic fiber (Mecham, 2018; Weinberger et al., 2019). In this condition, as there is gradual destruction of the alveoli, gas exchange is compromised, which can lead to aggravated airflow obstruction (Weinberger et al., 2019).

Although airflow obstruction has different origins in these two pathologies, patients often have the characteristics of both.

Beyond the damage caused to the lungs, COPD may be associated with extrapulmonary dysfunctions (Jaitovich & Barreiro, 2018). For example, it has been shown that modifications in brain function may be caused by COPD due to airway obstruction, hypoxia, and inflammatory mediators (M. Yin et al., 2019). Furthermore, changes and destruction of lung tissue increase the vulnerability of patients with COPD to develop heart diseases (André et al., 2019). COPD also contributes to skeletal muscle dysfunction that causes high rates of hospitalizations. This dysfunction arises from muscle atrophy and changes in fibers, metabolism, and anatomy (Jaitovich & Barreiro, 2018).

2.1.2 Causes and Symptoms

COPD is a complex and heterogeneous disease that can be caused by multiple factors such as smoking, environmental pollution, and genetic factors (Weinberger et al., 2019). Currently, smoking is a common cause of death by COPD (Kim et al., 2019). In 2030, 27% of deaths from COPD will be caused by smoking (Anzueto et al., 2015). Smoking causes serious damage to the bronchi, bronchioles, and pulmonary parenchyma. Besides that, there is overproduction of mucus and development of inflammatory processes mediated by inflammatory cells (leukotriene B_4, interleukin-8, and tumor necrosis factor-α) that contribute to tissue degradation (Weinberger et al., 2019). These factors and the release of reactive oxygen species (ROS) contribute to the progression of the disease. Due to smoking habits, alterations in the structure of the bronchioles (small airways) and the appearance of fibrosis occur. Consequently, it severely hinders the passage of air, which in COPD patients is worrisome (Weinberger et al., 2019).

Environmental pollution has a major impact on public health. It is considered an important risk factor that can cause or aggravate COPD (Jiang et al., 2016). The main risk factors associated with COPD are prolonged exposure to industrial pollution, traffic, and the combustion of fuels (Manisalidis et al., 2020). Exposure to air pollutants may also cause exacerbations of the disease and increased COPD morbidity and mortality (Manisalidis et al., 2020). In fact, air pollutants, such as carbon monoxide, nitrogen oxide, sulfur dioxide, ground-level ozone, and particulate matter pollution, have adverse effects on this disease (Jiang et al., 2016). Moreover, volatile organic compounds, dioxins, and polycyclic aromatic hydrocarbons also contribute to the worsening of the disease (Manisalidis et al., 2020).

According to the literature, genetic factors also contribute to the development of COPD (Weinberger et al., 2019). Deficiency in the α_1-antitrypsin glycoprotein is the most well-known genetic factor that causes COPD (Silverman, 2020). As stated by Weinberg and collaborators, α_1-antitrypsin is produced in the liver and circulates in the blood. In normal conditions, α_1-antitrypsin is an enzyme that inhibits the action of

serine proteases, protecting the lungs from their harmful effects (for example, emphysema with shortness of breath, coughing, and wheezing) (Wise & Hopkins, 2020). This protein is encoded by the SERPINA1 gene. When this gene is altered, there are modifications in the protein's structure, in its production, and its liberation. People with this genetic inheritance have reduced levels of α1-antitrypsin in the blood, and the protein may have an abnormal structure, which induces dysfunctions. The ZZ genotype is considered the most important form of α1-antitrypsin deficiency and is highly associated with premature development of emphysema (Weinberger et al., 2019).

In addition to the causes mentioned, COPD may also be associated with advancing age. Despite not being a fully clarified subject, successive damage throughout life due to exposure to agents and the aging of the airways and lung parenchyma can contribute to COPD. Furthermore, there is a propensity for the development of COPD when lung infections occur in childhood, and the factors that affect lung growth from birth to adolescence also contribute to increase the probability of developing this disease (GOLD, 2021).

Symptoms caused by COPD have a major impact on patients' life quality and well-being (Vogelmeier et al., 2020). According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD), dyspnea is the symptom that is most evident in COPD (GOLD, 2021). This disabling symptom is characterized by difficulty in breathing and is associated with anxiety (Anzueto & Miravitlles, 2017; Vogelmeier et al., 2020). Usually, the first symptom that appears in COPD is chronic cough (GOLD, 2021). Other symptoms, such as prolonged sputum production, are associated with inflammatory mediators that are indicative of bacterial exacerbation. In situations of aggravated illness, it may be related to fatigue, anorexia, and loss of weight. Depression also appears to be associated with COPD, which contributes to worsening health status and greater susceptibility to exacerbations and hospitalizations (GOLD, 2021; Vogelmeier et al., 2020).

2.1.3 Epidemiology in the World, Europe, and Portugal

Nowadays, it is estimated that 328 million people suffer from COPD worldwide. This illness is considered a *"silent killer"* in low- and middle-income countries (LMICs), accounting for over 90% of deaths (Quaderi & Hurst, 2018). A rise in COPD is expected over the next 40 years due to the continuous exposure to risk factors, increase in smoking in developing countries, and the aging of the world population. Even more serious is that, in 2060, there may be more than 5.4 million annual deaths from COPD (GOLD, 2021).

In the past, COPD was predominantly a disease that affected men (Aryal et al., 2014). However, nowadays the prevalence of COPD is equal in men and women. This change may be due to the increase

in tobacco consumption by women (GOLD, 2021). In addition, women's emancipation and cultural changes have led to women being more exposed to risk factors (Aryal et al., 2014). In terms of susceptibility, some studies report that women are more susceptible than men (GOLD, 2021). However, the relationship between genre, COPD, and susceptibility is complex, since biological and hormonal mechanisms can influence the way the disease manifests itself (Aryal et al., 2014).

In Europe, studies on COPD are scarce and there is a lack of knowledge about the disease (Gibson et al., 2013). A study carried out in 19 European countries on people over 40 years of age estimates that there is a prevalence of COPD of 12.4%. However, this percentage may not be correct due to the absence of information and irregularities in the distribution of data across Europe (Blanco et al., 2018). Another study estimates that, in Europe, there is a predominance of COPD between 4% and 10% (Miravitlles et al., 2016). According to the European Lung Foundation (ELF) and the European Respiratory Society (ERS), prolonged exposure to risk factors in the workplace is responsible for 15% to 20% of cases. Recent studies reveal that, in the European Union, the prevalence of COPD in men and women was not significantly different, which demonstrates an increase in the prevalence of COPD in the female population. In addition, there is evidence that COPD is a critical health problem for women, with more and more cases of COPD among women (Ntritsos et al., 2018).

According to the Portuguese Lung Society (Sociedade Portuguesa do Pulmão - SPP), in Portugal, COPD is one of the main causes of death, affecting approximately 800 thousand people. In addition, it has a major impact on public health and is considered the chronic respiratory disease with the highest mortality rates. It is estimated that one in seven Portuguese over 40 years of age has COPD. In 2016, 20.7% of deaths from respiratory disease were caused by COPD (SPP, 2019). In 2017, it was responsible for 2 627 deaths, representing 2.4 % of total deaths in the country. The disease affected more men than women, and about 95% of deaths occurred in people over 65 years (INE, 2019). In 2018, there were 2 834 deaths, and the mortality increased by 7.9 % compared to the previous year (INE, 2020). In Portugal, COPD cases have been on the rise and, as this disease is underdiagnosed, the number of people who have COPD may be much higher (Munhá, 2020). Besides, the diagnosis is often made late and, consequently, when patients turn to the doctor, the disease is already at a very advanced stage. Thus, it is extremely important to detect the disease in the early stages, which will allow to control and slow the disease progression. For this, it is necessary to raise awareness and inform individuals about the existence of COPD, its associated risk factors, and associated symptoms (Simão & Carvalho, 2018). The truth is that this chronic respiratory disease is not given due importance despite its severity. It is urgent that

governments, industries, and the population acquire new strategies for prevention, reduction of risk factors, and delaying the progression of the disease (Gibson et al., 2013).

2.1.4 Exacerbations

COPD exacerbations are complex events responsible for the acute worsening of respiratory symptoms, contributing largely to disease progression and impaired lung function (GOLD, 2021; Oliveira et al., 2018). Besides the need for additional therapy, exacerbations have a major impact on the economic level, on the quality of life of patients, are responsible for high rates of hospitalizations, worsening comorbidities, and a consequent increase in mortality and morbidity rates (Oliveira et al., 2018; Shimizu et al., 2015; van Bragt et al., 2020). Exacerbations negatively influence the development of the disease because they intensify the inflammation of the airways, restrict the passage of air, and induce an increase in mucus production which leads to the worsening of dyspnea, which is the most common symptom in COPD and the key symptom when an exacerbation occurs (GOLD, 2021).

Given that exacerbations have a negative impact and contribute to the development and worsening of the disease, their treatment is essential, having as main objectives the prevention and mitigation of their effects, and possible consequences. According to the GOLD, exacerbations can be classified as mild, medium, and severe. Often, severe COPD exacerbations are referred as acute (AECOPD). Mild exacerbations can be treated using short-acting bronchodilators (SABDs). The use of corticosteroids, SABDs plus, and antibiotics are indicated for the treatment of medium exacerbations. However, in the case of severe exacerbations, which are associated with acute respiratory failure, medical monitoring and hospitalization is also strictly necessary. Recurrently, when an acute exacerbation occurs, the prognosis is critical and there is an increased risk of death (GOLD, 2021).

Exacerbations tend to become more severe and more frequent as COPD progresses (Bouquet et al., 2020). In addition, they are considered heterogeneous due to the variation of the phenotype, etiological factors, degree of severity, and response to treatment (Oliveira et al., 2018; Pavord et al., 2016). The high amount of eosinophils in the blood can trigger the appearance of exacerbations, as well as limited changes in the inflammatory profile, air pollution, and comorbidities. Furthermore, poor compliance to maintain therapy and a decrease in the average temperature of the environment may also lead to exacerbations (Oliveira et al., 2018). Most exacerbations are related to respiratory tract infections and are caused by interactions between pathogens, the lung environment and the associated host response, which lead to increased airway inflammation (Leung et al., 2017; Short et al., 2021). In exacerbations, infections can be caused by bacterial or viral colonization, as well as by viral and bacterial co-infection, having a greater impact on the inflammatory response of the airways (Su et al., 2018).

The development of molecular techniques made possible the detection and identification of microorganisms and allowed us to understand the importance of bacteria and viruses in exacerbations. The main viruses involved in infections that can lead to exacerbations are the Influenza virus, the human Rhinovirus (HRV), Respiratory syncytial virus (RSV), and Coronavirus (Bouquet et al., 2020). Since the focus of this thesis are bacterial related exacerbation, the role of virus will not be further detailed.

Bacterial infections play an extremely important role in the appearance and development of the pathogenicity of exacerbations (Ritchie & Wedzicha, 2020). It is important to refer that, in COPD, 50% of exacerbations are associated with bacterial infections. Studies carried out with sputum samples and molecular techniques show an evident prevalence of bacteria when an exacerbation occurs (Saxena et al., 2016). Moreover, based on traditional culture techniques, it has been shown that, in 40% to 60% of exacerbations, bacterial isolates are present (Ritchie & Wedzicha, 2020). NTHi is the isolated bacterium that most causes bacterial lung infections in COPD and is often associated with exacerbations (Short et al., 2021). *M. catarrhalis*, *S. pneumoniae*, *S. aureus, P. aeruginosa*, *K. pneumoniae* are also prevalent when exacerbations occur (Leung et al., 2017; Su et al., 2018). Indeed, rich and complex communities of bacteria are normally present in the lungs of both healthy individuals and those with COPD. However, when there is an exacerbation, changes occur in these microbial communities, which disturb the dynamic balance between the bacteria that normally exist in the pulmonary microbiome (lung microbiota dysbiosis) (Ko et al., 2016; Ritchie & Wedzicha, 2020; Su et al., 2018). Thus, the acquisition of new bacterial strains is one of the main factors that trigger a period of exacerbation, and the changes in species, and bacterial load are extremely important characteristics in the progression of exacerbations, because they induce airway inflammation and contribute strongly to the decline in lung function (Leung et al., 2017; Short et al., 2021).

2.2 *Haemophilus influenzae*: a Relevant Bacteria in COPD Exacerbations

H. influenzae is a coccobacillus, Gram-negative, facultative anaerobic and commensal bacterium. It is restricted to humans, is commonly present within their nasopharyngeal flora, and requires hemin and nicotinamide adenine dinucleotide (NAD) for growth (Musher, 1996; Short et al., 2021). It is an opportunistic bacterium, since it can become pathogenic, and its mode of transmission occurs through direct contact with respiratory secretions or respiratory droplets. Moreover, it is a major cause of AECOPD, but it is also associated with other conditions such as otitis media and pneumonia (Short et al., 2021). The classification of *H. influenzae* is based on the presence or absence of a polysaccharide capsule. When a capsule is present, *H. influenzae* can be divided into six serotypes, from “a” to “f”. Strains of *H.*

influenzae that do not have a polysaccharide capsule are called non-typlable *Haemophilus influenzae* (NTHi) (Sriram et al., 2018).

NTHi has great relevance in COPD. When these bacteria are present in the lower airways of patients with COPD, they reveal high pathogenicity, being associated with states of considerable inflammation with consequent worsening of symptoms and increased frequency of exacerbations (Sriram et al., 2018). In fact, it is the most frequently isolated bacterial pathogen, being detected for long periods of time in 30% of COPD patients during stable disease. Furthermore, it is the main cause of bacterial exacerbations, being associated with 30% of AECOPD (Short et al., 2021; Sriram et al., 2018).

The pulmonary vulnerability and fragility in patients with COPD facilitates the colonization of the airways by bacteria such as NTHi. Hypersecretion of mucus is an important physiological feature, since the dehydrated and thick mucus secretions combined with a reduced frequency of the ciliary beat severely affect the lung's primary innate defense mechanism, allowing the colonization by NTHi (Bustamante-Marin & Ostrowski, 2017; Short et al., 2021). Persistent airway infection, caused by NTHi, largely contributes to chronic airway inflammation, and accelerates the loss of lung function. The development of several mechanisms that allow NTHi to establish infection in the hostile airway environment, discussed below, allows this pathogen to remain in the airways from months to years (Ahearn et al., 2017). The use of this variety of mechanisms causes NTHi to overcome the innate and adaptive defenses of the host, and, in addition, makes treatment with antibiotics ineffective (Short et al., 2021).

The main virulence mechanisms that contribute to persistent infection are adhesion, invasion of host cells, and biofilm formation (Ahearn et al., 2017). The first stage of a bacterial infection involves the initial adhesion of the bacteria to the host cells (Short et al., 2021). The adhesion virulence mechanism is crucial for the initial colonization of the lower airways and the progression of persistent infection. The adhesion of NTHi to the respiratory cell epithelium and to the proteins of the extracellular matrix is mediated by high molecular weight adhesins HMW1, HMW2, protein E, and protein F (Sriram et al., 2018). Other key adhesins present in NTHI, such as type IV Pilus (TfP), and outer membrane proteins OMP P1 and P5, together with HMW1/2, in addition to facilitating the initial attachment to the substrate, facilitate the attachment to mucins present in the mucus of the host (Weeks et al., 2021). As shown in Figure 1, the adhesins TfP, OMP P1, and OMP P5 bind to the ICAM-1 and CEACAM1 receptors (Figure 1-A,B). In addition, an unknown adhesin binds to the β-glucan receptor (βGR) (Figure 1-C), the phosphocholine adhesin binds to the platelet activating factor receptor (PAFR) (Figure 1-D), and the protein E adhesin binds to vitronectin (Figure 1-E) (Ahearn et al., 2017).

When the host fails to eliminate the invading bacteria, invasion occurs. Invasion can occur when the bacteria are engulfed by host respiratory cells, when they remain on the surface of the respiratory epithelium, or when they are bound by other host factors such as fibronectin, enhancing biofilm development (Short et al., 2021). In fact, NTHi evolved strategies for intracellular survival within lysosomes (Figure 1-F). The success of colonization and invasion by NTHi is also due to its ability to avoid recognition and elimination by immunological action (they escape the neutrophil extracellular traps (NETs) (Figure 1-G) and, furthermore, to its capability to modify gene expression, to present antigenic variation on the surface, and to bind to host serum factors, thus conferring serum resistance (Ahearn et al., 2017).

The development of biofilms (Figure 1-G) is a commonly used strategy of bacterial persistence inside the respiratory system, which is characterized by changes in bacterial behavior, such as a reduction of cellular metabolism and the production of obstructive extracellular polymeric substances (EPS) (Figure 1-G) (Ahearn et al., 2017; Short et al., 2021). With greater resilience to environmental stress and a higher tolerance for antibiotics, biofilms provide a protective environment against immune cells and predation by other microorganisms (Melton & Anderson, 2019).

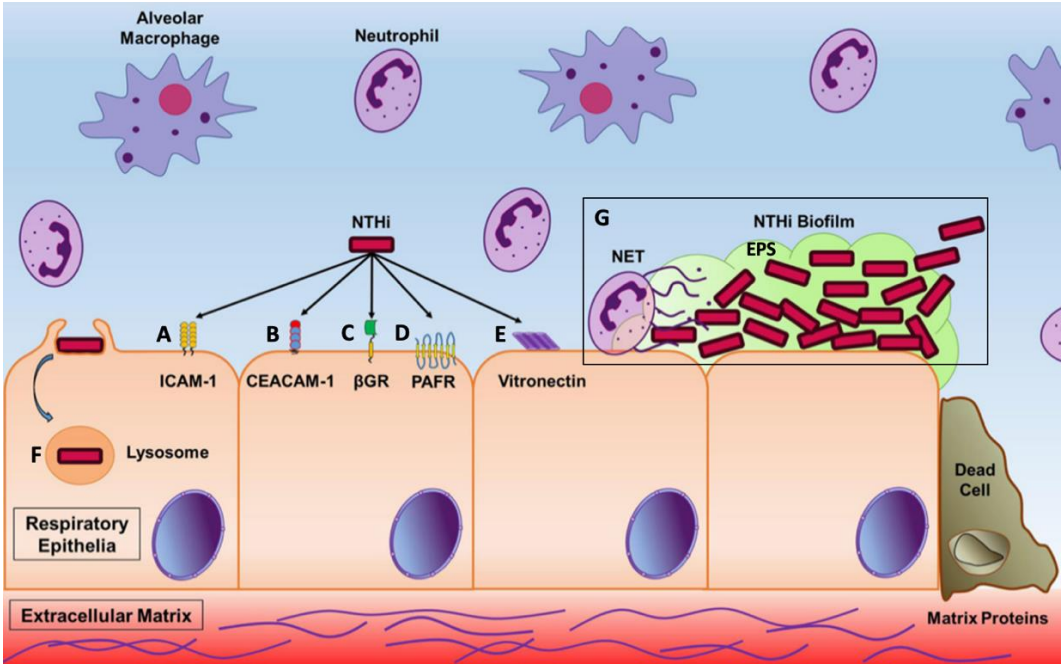

Figure 1 - Mechanisms that allow NTHi to establish infection in the hostile environment of the airways. The binding of NTHi to respiratory epithelial cells is mediated by adhesins. Adhesins bind to cell surface receptors: ICAM-1(A), CEACAM-1(B), β-glucan receptor (βGR) (C), platelet activating factor receptor (PAFR) (D), and vitronectin receptor (E). NTHi also developed mechanisms to survive intracellularly within lysosomes (F). Intracellular invasion occurs when NTHi is directly linked to the respiratory epithelium. The formation of NTHi biofilm is possible due to the bacterium's binding to the pulmonary epithelium, EPS production and NET resistance (G) (adapted from Ahearn (2017)).

2.3 Biofilm Formation and Bacterial Communication System

Biofilms are considered a special adaptation that facilitates bacterial colonization and provides bacteria the ability to persist, resist host defenses, and evade the effect of antibiotics (Murray et al., 2016; Welp & Bomberger, 2020). This mechanism of bacterial persistence, composed of complex microbial communities, is frequently present in the human respiratory tract and, in COPD, is involved in the vicious cycle of infection and inflammation that leads to disease progression (Blasi et al., 2016; Short et al., 2021; Welp & Bomberger, 2020).

Generally speaking, biofilms are a complex structure of well-organized bacterial communities attached to a suitable surface (biotic or abiotic) surrounded by a self-produced matrix of EPS (Jorge et al., 2019; Meroni et al., 2021; Pirrone et al., 2016). This polymeric matrix connects the cells to each other, makes the connection to the surface, and is composed of 97% w/w of water with polysaccharides, proteins, lipids, and extracellular DNA (eDNA) (Melton & Anderson, 2019; Murray et al., 2016). Environmental stressors include UV radiation, desiccation, nutrient depletion, low nutrient availability, extreme pH, high temperature, high salt concentrations, high pressure, and antimicrobial agents cause bacteria to form biofilms (Muhammad et al., 2020). Its formation usually includes five stages: (I) reversible attachment to a suitable surface; (II) irreversible attachment; (III) proliferation of biofilm; (IV) maturation; and (V) detachment of bacterial cells (Figure 2) (Pinto et al., 2020).

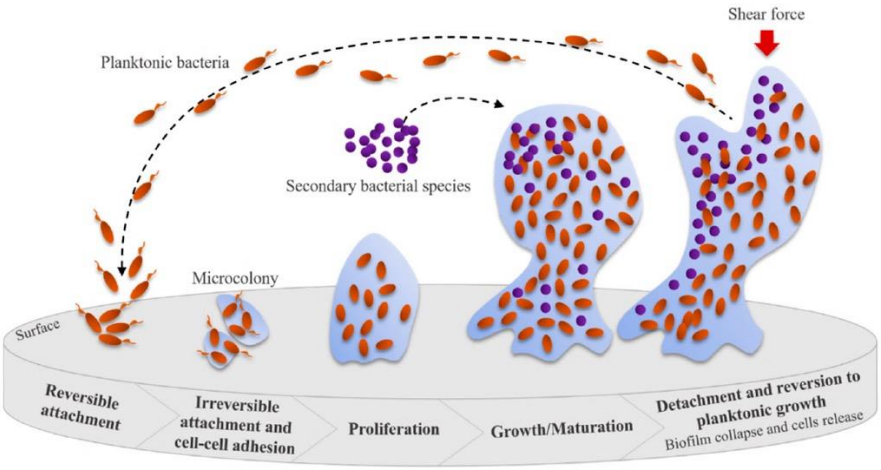

Figure 2 - Biofilm formation cycle (Pinto et al., 2020).

The first stage begins with the reversible attachment of planktonic bacteria to a surface with the establishment of interactions guides (hydrophobic interactions, electrostatic forces and van der Waals interactions). Depending on the microorganism, this stage is mediated by secreted adhesins, flagella, and other surface molecules. Then, irreversible attachment begins due to the production and secretion of EPS by bacteria and specific adhesins, such as (TfP), OMPs, and HMWs adhesins on NTHi (Meroni et al.,

2021; Pinto et al., 2020; Weeks et al., 2021). After that, the proliferation of the biofilm structure begins, and then the biofilm grows (weight and width) and matures. In the stage of maturation, water passage channels, with the capacity to transport nutrients to the microbial communities and remove toxic products, are formed (Meroni et al., 2021; Muhammad et al., 2020; Pinto et al., 2020). Furthermore, within the biofilm, there is the development of microenvironments resulting from the establishment of oxygen and nutrient gradients, which make bacterial cells appear stratified and with different phenotypes depending on their spatial organization at the core of the biofilm or near the outer surfaces (Jorge et al., 2019; Melton & Anderson, 2019). In the final stage, bacterial cells initiate the process of detachment and the biofilm collapse. This process can be induced in response to changes in their environment (antimicrobial stress, matrix-degrading enzymes, nutrients starvation) in which the bacteria initiate detachment by themselves (active action), or they can initiate detachment due to external forces (e.g., sloughing, erosion, external organism interaction, and fluid shear) (passive action). Environmental changes such as alterations in temperature, pH, oxygen deficiency can also be factors that contribute to biofilm dispersion in active action (Melton & Anderson, 2019; Muhammad et al., 2020). In this terminal process, the cells move and disperse until they find a suitable substrate to start a new cycle of biofilm formation (Pinto et al., 2020).

When living in community, bacteria have a very special way of communicating with each other through quorum sensing (QS). This is a cell-to-cell biochemical/molecular communication system performed by specific molecules called autoinducers (AIs) or QS signals. These biochemical signals are produced by the bacteria themselves, secreted in the extracellular environment, and detected by the local population, thus mediating communication between the same species or between different species. In addition, they are responsible for controlling population density, gene expression and various functions depending on the environment (Table 1) (Grandclément et al., 2016; Pinto et al., 2020). For example, in the case of biofilm formation, a sufficient number of bacteria, the quorum, is required in order to be formed. Each bacterium present in the medium will produce a specific molecule and, when the number of bacteria present is favorable, the concentration of the molecule will be sufficient for the coordination and expression of the genes to support the colony. At that moment, when the bacteria determine that the size of the colony is large enough, the biofilm is formed (Murray et al., 2016).

Table 1 - Functional categories controlled by QS (Grandclément et al., 2016).

Functional categories	Examples
Cell maintenance and proliferation	Exoenzymes production, siderophores synthesis, sporulation, acid resistance
Cell behaviours	Biofilm formation and dispersal, motility, adhesion
Horizontal gene transfer	Plasmid conjugation, competence
Interactions with host and other microbes	Virulence factors, exopolysaccharide production, bioluminescence, antibiotics, host colonization factors

The properties of autoinducers and the response they induce in coordinating population behaviors ensure bacterial survival and propagation in natural environments where a variety of bacterial species coexist (Federle & Bassler, 2003; P. Smith & Schuster, 2019). Intraspecies and interspecies communication is carried out by autoinducers (Sheela et al., 2019). In intraspecies communication, only the species of bacteria that produces the autoinducer can detect and respond to it. Intraspecies QS has differences between Gram-positive and Gram-negative bacteria. In Gram-negative bacteria, the QS system specifically uses the acyl-homoserine lactone (AHL) autoinducer. AHL is produced and freely diffused into and out of the cell and only members of the same species recognize and respond to the peptide. In Gram-positive bacteria, QS is mediated by oligopeptides or autoinducing peptides (AIPs) that are transported to the extracellular medium via oligopeptide transporters because the bacterial membrane is not permeable to AIPs. This type of communication with bacteria of the same species is relevant when they live together with several species of bacteria and in an environment with similar chemical structures, as it allows them to distinguish themselves from other species, assess their numbers, and coordinate particular behaviors of the species. In addition to the species-specific QS communication system, all bacteria have the interspecies QS communication system (Federle & Bassler, 2003). Autoinducer-2 (AI-2) is one of the main signaling molecules in the bacterial QS process with the ability to control many processes, such as the production of virulence factors, biofilm formation and motility. These molecules are produced by Gram-positive and Gram-negative bacteria and participate in the regulation of gene expression and physiological behaviors of bacteria in interspecies communication (Armbruster et al., 2011; Sheela et al., 2019). This type of communication is evident when some species cannot produce their own autoinducers (AI-2), however they have receptors for the autoinducers of other species (Windsor, 2020). Bacteria can also produce AI-2 through protein synthesis, but it is not detected by the receptor and can be used to regulate physiological behaviors of other bacteria (J. Zhao et al., 2018).

QS communication plays a facilitating role in several functions, such as protection from toxins, nutrient starvation response, competition with other bacteria for resources and limited space, and

survival, as well as establishing symbiosis (Nadell et al., 2016; Sheela et al., 2019). In bacterial cooperation, production by bacteria of extracellular products, costly to produce and shareable for the entire population (public goods) coordinated by QS, can benefit neighboring cells in biofilm communities (K. Zhao et al., 2019). Examples of public goods include extracellular enzymes important for nutrient digestion, biosurfactants that promote bacterial motility to the substrate, and toxins that damage host tissue to obtain nutrients. Other examples of public goods are exopolysaccharides that provide structure and protection to biofilms, siderophores that capture iron from the environment, as well as AIs described above (P. Smith & Schuster, 2019). The production of molecules by bacteria are not always considered public goods and may establish competitive relationships. For example, bacterial QS can be blocked by inhibiting the production, delivery, or detection of AIs. Blockade of QS is performed by enzymes, such as lactonases or acylases, which inactivate the AI and consequently interrupt cell-to-cell communication. Other competitive relationships relate to competition for nutrients mainly for iron, production of antibiotics by bacteria (bacteriocins), such as piocin, and production of molecules to kill or interfere with microbial growth, as well as transfer of toxins by physical contact (Welp & Bomberger, 2020).

2.4 Biofilms in COPD

Chronic respiratory diseases, including COPD, are often associated with the formation of polymicrobial biofilms (Scoffield & Wu, 2019; Welp & Bomberger, 2020). Polymicrobial biofilms are complex and dynamic mixed species communities that are constantly evolving (F. Harrison et al., 2020; Welp & Bomberger, 2020).

Patients with COPD have the ideal conditions for biofilm formation due to impaired ciliary clearance system and inflammatory hyperplasia of mucus secretion. NTHi is the bacterium that initiates the process of colonization and attachment to respiratory epithelial cells. In human lungs, this microorganism can survive in the mucus-rich environment and has available hemin and NAD essential for its survival. It has an arsenal of adhesins, such as type TfP and OMP P1, that bind to the ICAM-1 protein and the CEACAM-1 glycoprotein located on the host cell surface (Figure 1) that allow the adhesion and invasion of lung cells (Figure 3). The production and development of EPS by aggregates of NTHi allows the entry of new bacterial species. The polymicrobial consortium develops microcolonies with water and oxygen channels due to the development of complex intraspecific and interspecific interactions, changes in metabolism and gene expression that drive the water and oxygen gradient. The quorum sensing system allows the dispersion of the biofilm with consequent dissemination and colonization of new sites. This cycle causes

the infection to persist and become chronic, leading to tolerance and resistance to antibiotics and to the host's immune system (Weeks et al., 2021).

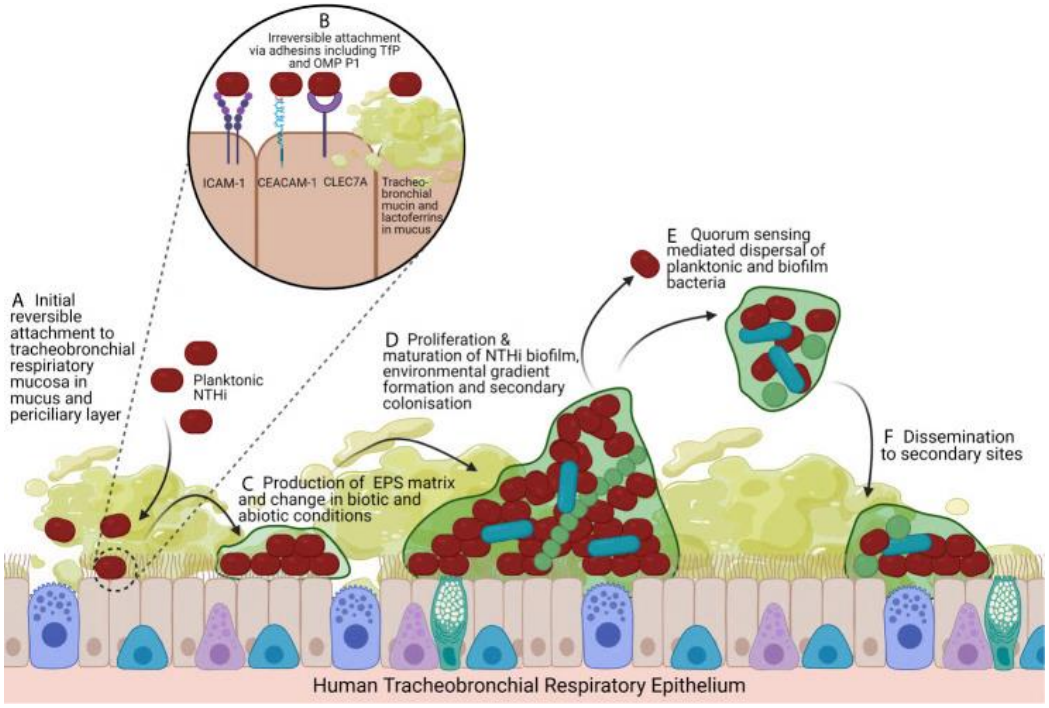

Figure 3 - Life cycle of biofilm in COPD. (A) Biofilm formation begins with the reversible attachment of NTHi to a suitable surface of the air-liquid interface of the tracheobronchial respiratory mucosa composed of columnar epithelium predominated by ciliated epithelial cells interspersed with secretory cells and basal cells. (B) Irreversible fixation occurs due to the presence of NTHi TfP and OMP P1 adhesins that bind to specific proteins (ICAM-1, CEACAM-1, CLEC7A) present in respiratory epithelial cells. NTHi also has adhesins that bind to mucus proteins such as mucin and lactoferrin. (C) The production of EPS by NTHi favors the acquisition of secondary colonizers. (D) Biofilm develops with the entry of secondary colonizers. The species present initiate complex intraspecific and interspecific interactions, and changes occur in gene expression and metabolism. As a result, nutrient and oxygen gradients are formed, and bacterial differentiation takes place. The biofilm matures and nutrient and water channels are formed. (E) Bacteria disperse in planktonic form or in biofilm due to the quorum sensing system. (F) The process ends with the colonization of suitable substrates (Weeks et al., 2021).

In a polymicrobial biofilm, ecological interactions, for example, synergism, commensalism, mutualism, competition, etc., between bacterial species or strains are extremely important for the functioning of the biofilm ecosystem, as well as for the expression of virulent or persistent phenotypes. The diversity of the polymicrobial lung microbiome may favor interspecific interactions between bacteria. These interspecific interactions can be indirect due to biotic and abiotic changes that favor the proliferation of secondary colonizers in airway diseases. For example, NTHi infection increases inflammation that can result in microbial changes and COPD exacerbations. Furthermore, is also increases airway obstruction and the production of a viscous substrate by upregulating MUC2, which favors the secondary colonization of bacteria over time (Weeks et al., 2021). Interspecific interactions can also be more direct when there is the establishment of multi-species biofilms that cooperate in substrate adhesion and stabilize the biofilm

structure, such as the multi-species biofilms of NTHi and *S. pneumoniae* that together produce and share an EPS. NTHi and *S. pneumoniae* often co-colonize the respiratory tract of patients with COPD, interacting synergistically, promoting initial adhesion to the substrate, biofilm formation, and survival. Thus, together, these bacteria develop an EPS matrix made up of TfP, LOS, eDNA, QS signals, proteins, and carbohydrates essential in cooperative adhesion and stability of the biofilm structure (Kyd et al., 2016; Weeks et al., 2021). In addition, *H. influenzae* produces β-lactamases that protect *S. pneumoniae* from treatment with β-lactams. However, they also establish a competitive relationship because, as *S. pneumoniae* develops, the pH of the biofilm decreases and there is production of a biocide, hydrogen peroxide, which kills NTHi (Kyd et al., 2016). Despite killing NTHi, the production of this biocide in the long term stimulates the production of neutrophils that together make a selective pressure of persistent and ROS-tolerant strains of NTHi. Furthermore, hydrogen peroxide is a source of nutrients and DNA due to bacterial death and senescent host cells that maintain the biofilm (Weeks et al., 2021).

M. catarrhalis, responsible for 10% of exacerbations in COPD, also favors NTHi colonization after infection due to biotic changes. Specifically, its binding to epithelial cilia reduces the frequency of their beats, compromising mucociliary clearance with consequent formation of mucus plugs that are colonized by NTHi. In otitis media (OM) models, the highly active catalase production by *M. catarrhalis* protects NTHi from the bactericidal effect of *S. pneumoniae* hydrogen peroxide (Bair & Campagnari, 2020). The presence of *M. catarrhalis* in the COPD lung could have the same effect.

As interactions play an important role in biofilm stability, treatment of polymicrobial biofilm infections should be focused on these interactions (F. Harrison et al., 2020). COPD prevention and treatment may require understanding microbial interactions that modify diversity and the microbial community (Welp & Bomberger, 2020). However, many of the microbial interactions in the respiratory tract are still unknown because the presence of mixed species in chronic infections is often confirmed through PCR and sequencing. These techniques do not consider the spatial organization of the bacterial community, so crucial information about the composition of the biofilm aggregate, the spatial organization, and the possible interactions between different species may be lost (Kvich et al., 2020). Recently, two innovative methods for the rapid diagnosis of *H. influenzae* biofilms were created. One of the methods is the identification of *H. influenzae* through molecular imaging in real time due to the use of an environmentally sensitive fluorophore 7-nitrobenz-2-oxa-1,3-diazole conjugated with polymyxin which fluoresces in contact with the lipid A component of Gram-negative bacteria. The other method involves similar smart probes capable of identifying cellular metabolites. Combined, these two methods may provide a selective labeling of molecules associated with *H. influenzae* biofilms, enabling their rapid

diagnosis in COPD lung and even in other conditions. Although there are studies of NTHi biofilms and their persistence during infection, they come from OM models and little information exists on these biofilms in COPD (Short et al., 2021). More research on NTHi biofilms is needed in order to characterize the biofilm formation capacity and the bacteria-host interactions that will allow selecting anti-biofilm therapeutic targets helping in the diagnosis and treatment of COPD patients (Weeks et al., 2021).

2.5 Antimicrobial Resistance (AMR)

AMR is an emerging problem worldwide, contributing strongly to deadly bacterial infections (Jorge et al., 2019). In 2019, it is estimated that AMR was responsible for at least 1.27 million deaths worldwide and by 2050 the number is predicted to increase dramatically to 10 million annual deaths (O'Neill, 2016). AMR is a natural process in which microorganisms (such as bacteria, fungi, viruses and parasites) develop defense mechanisms to counteract the lethal effects of antimicrobials (e.g. antibiotics, antifungals, antivirals, antimalarials) giving rise to multidrug-resistant organisms (MDR) or "superbugs" (Aslam et al., 2018; CDC, 2021; WHO, 2017). However, the excessive or inappropriate use of antimicrobials accelerates the AMR process, which leads to the dissemination of these microorganisms and their resistance mechanisms (WHO, 2017).

According to the WHO, antibiotic resistance is, today, one of the greatest threats to public health, development, and food security (WHO, 2020). Although antibiotics play a crucial role in the treatment of infections, due to their overuse and inadequate use, it is becoming a problem worldwide (Jorge et al., 2019). Worryingly, if antibiotics lose their effectiveness altogether, the ability to treat infections and public health will be compromised (CDC, 2020). The main causes of the emergence of antimicrobial resistance are: (1) the excessive and unnecessary use of antibiotics in livestock, fish farming, and agriculture; (2) overconsumption of antibiotics due to medical overprescription, self-medication, incorrect antibiotic use, and over-the-counter accessible antibiotics; (3) lack of standard guidelines for antibiotic use; (4) poor infection control in hospitals and clinics; (5) lack of rapid laboratory tests; (6) lack of development of new antibiotics; (7) lack of hygienization and sanitation practices; (8) access to counterfeit drugs; (9) release of unmetabolized antibiotics or their residues into the environment (Aslam et al., 2018; CDC, 2019b; Jorge et al., 2019).

There are four general mechanisms of bacterial resistance, which are (1) limited uptake of a drug, for example in gram-negative bacteria, the limitation of drug permeability due to the presence of an outer membrane, or by mutations in the number, type and size of porins present in the outer membrane, which may be restricted the entry of hydrophobic antibiotics such as β-lactams, fluoroquinolones, tetracyclines,

and chloramphenicol. In gram-positive bacteria, such as *S. aureus*, there is greater peptidoglycan synthesis with more D-Ala-D-Ala residues that bind to the antibiotic vancomycin, preventing its binding to the target site (C Reygaert, 2018; Christaki et al., 2019; Lowy, 2003); (2) drug target modification. Changes occur in the antibiotic binding targets present in bacterial cells, due to mutations, such as changes in penicillin binding proteins (PBP) that confer resistance to β-lactam antibiotics or by enzymatic action, such as 23SrRNA methylation that confers cross-resistance to macrolides, and lincosamides (C Reygaert, 2018; Christaki et al., 2019); (3) drug inactivation by degradation. For example, the destruction of the antibiotic by enzymes, such as β-lactamases, or by the transfer of a chemical group, called transferases (e.g., acetyl, phosphoryl, and adenyl groups) (C Reygaert, 2018); and (4) drug efflux systems with the function of pumping toxic molecules out of the cell to regulate the internal bacterial environment (Christaki et al., 2019). In addition, antibiotic resistance can also be performed through mechanisms of horizontal gene transfer through plasmids, transposons or integrons or being an integral part of bacterial chromosomal DNA (M. Kyd et al., 2011).

Recently, there has been an increasing development of bacterial resistance to the main antibiotics used to treat infections (Aslam et al., 2018; Short et al., 2021). This resistance compromises the effectiveness of the treatment and leads to the selection of resistant subpopulations that can make COPD exacerbations even more persistent and severe. For example, in exacerbations, the overuse and widespread use of antibiotics for the treatment of patients in primary and secondary health care contributes largely to the resistance of bacteria (Beasley et al., 2012; CDC, 2019a; Short et al., 2021).

Antibiotic treatment is not necessary in all patients with COPD and avoiding its unnecessary use is important to limit the development of antimicrobial resistance (MacLeod et al., 2021). Antibiotics should be prescribed in case of moderate or severe exacerbation and, according to the GOLD parameters, patients should have three symptoms: increased dyspnea, sputum volume, and sputum purulence; having two symptoms, one of which is increased sputum purulence; or needing ventilation (invasive and non-invasive) (GOLD, 2021; Nissly & Prasad, 2014; Siddiqi & Sethi, 2008). The main antibiotics used in these situations are the advanced macrolides (azithromycin, clarithromycin), ketolide (telithromycin), cephalosporin (cefuroxime, cefpodoxime or cefdinir), doxycycline, trimethoprim/sulfamethoxazole, fluoroquinolone (moxifloxacin, gemifloxacin, levofloxacin), and amoxicillin/clavulanate (Siddiqi & Sethi, 2008).

A systematic review to determine the prevalence, patterns, risk factors, and consequences of AMR in COPD revealed that at least one of the three bacteria *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* present in COPD patients exhibits high levels of resistance to at least one of the antibiotics

amoxicillin, doxycycline, and clarithromycin, which may contribute to treatment failure and emergence of resistance (D. Smith et al., 2022). According to GOLD, 2022, treatment of azithromycin and clarithromycin for one year in patients prone to exacerbations reduces the risk of exacerbations compared with usual care (GOLD, 2022). However, long-term treatment with macrolides, namely azithromycin and clarithromycin, can increase bacterial resistance to this type of antibiotics (Y. Cui et al., 2018). Macrolide resistance can be caused by modification of macrolide target sequences, as well as macrolide efflux pump systems (Djamin et al., 2020). In addition, ketolides can also be inhibited by the AcrAB efflux pump present in *H. influenzae*, and the overproduction of efflux pumps by *P. aeruginosa* in COPD contributes to resistance mechanisms acting on the inhibition of fluoroquinolones, β-lactams, aminoglycosides, and polymyxin B (M. Kyd et al., 2011).

Acquisition or expression of β-lactamases is a common resistance mechanism that causes β-lactamic ring destruction of β-lactam antibiotics. *H. influenzae*, *M. catarrhalis*, and *P. aeruginosa*, produce this enzyme and, therefore, the use of these antibiotics, such as amoxicillin, to treat COPD related infections is not recommended. In *H. influenzae*, inhibition of β-lactams occurs either through the production of β-lactamases or via the alteration of PBPs. Most strains only have one mechanism, but many strains have both and are known to be β-lactamase-positive amoxicillin clavulanate-resistant. Also, the alterations in porins, mentioned above, may be involved in the resistance mechanisms in COPD, where, for example, the alteration in porin 2 present in *H. influenzae* partially contributes to the resistance to ampicillin, penicillin, cephalothin, and chloramphenicol (M. Kyd et al., 2011).

In view of the resistance mechanisms expressed by bacteria, namely the production of β-lactamases, alteration of membrane permeability, efflux pump systems, and mutations in microbial targets, the selection of antimicrobial therapy for exacerbations is a challenge since it is necessary to obtain microbial clearance and avoidance of microbial stimulation (M. Kyd et al., 2011; Short et al., 2021). To circumvent this adversity, mucolytic agents are currently being prescribed on a large scale by clinicians as they decrease mucus viscosity and increase its fluidity, helping with microbial clearance (Papi et al., 2020; Weeks et al., 2021). Specifically, erdosteine can be used as an adjunct to therapy in COPD as it has a significant potentiating effect of antibiotics against chronic respiratory infections (Papi et al., 2020).

2.6 The Role of *H. influenzae* Biofilms in AMR

The formation of biofilms is a form of AMR and is considered a recurring problem due to their persistence in infections (Jorge et al., 2019; Welp & Bomberger, 2020). Biofilms protect bacteria and

make them tolerant to antibiotics, thus hampering the clinical treatment of a biofilm related infection (Melton & Anderson, 2019). In biofilms, due to poor growth conditions, it is common to find tolerance in resident bacteria. Tolerance can be defined as the ability of the microorganism to survive a brief exposure to high antibacterial concentrations. Therefore, the antibiotic takes longer to kill the bacteria. This happens because biofilms have a gradient of oxygen and nutrients, which makes the bacteria that are in the lower layers have less access to them. This phenomenon induces these bacteria to reduce their metabolic activity and enter a slow growth or state of dormancy, giving rise to the persister cell phenotype. When a bacterial subpopulation has a persistent phenotype, although the antimicrobial can eliminate the other cells, the subpopulation survives the treatment and can grow again, causing the infection to relapse. This persistence phenotype is one of the main causes of the inefficiency of some antimicrobials (Jorge et al., 2019). In fact, a proteomics study indicated that *H. influenzae* biofilms in a semi-dormant state have reduced cellular metabolism and protein synthesis (Post et al., 2014). This altered cellular activity enhances the limited diffusion of drugs through the biofilm ECM components, and the decreased protein synthesis minimizes the activity of protein synthesis inhibitor antibiotics, such as macrolides (Short et al., 2021).

In biofilms, the matrix that surrounds them also contributes to their high resistance/tolerance to external stresses, namely the action of antimicrobials and the immune system (Jorge et al., 2019). The production of a thick ECM by *H. influenzae* contributes to resistance to neutrophils and NETs, blocks macrophage access to biofilm cells, thus preventing phagocytosis, and aids in cleavage of immunoglobulin (IgA), which is considered a major component of the immune system and a first-line defense mechanism of the mucosal surface, via IgA proteases. The eDNA belonging to the ECM is also an important component of antibiotic resistance, because it stimulates biofilm formation and binds to human β-defensins present in greater amounts in COPD compared to healthy individuals, reducing their antimicrobial properties (Short et al., 2021).

H. influenzae is tolerant to imipenem and, in a biofilm community, this tolerance can be strengthened due to the differential behavior of biofilm cells, such as fluctuating gene expression and cell metabolism that can lead to the development of heteroresistant cell phenotypes and persistence (Short et al., 2021). Interestingly, β-lactam antibiotics, probably due to a stress response, induce the formation of *H. influenzae* biofilms. Furthermore, in polymicrobial biofilms, *M. catarrhalis* has the ability to secrete β-lactamases, which confer protection to *H. influenzae* from ampicillin treatment, and the signaling molecule AI-2 from *H. influenzae* induces resistance of *M. catarrhalis* to clarithromycin and trimethoprim-sulfamethoxazole (Jorge et al., 2019). The synergetic interaction of the *in vitro* polymicrobial biofilm of

both *H. influenzae* and *S. pneumoniae* can also promote protection from the effects of antibiotics, since the production of β-lactamases by *H. influenzae* and the production of ECM by both bacteria were able to protect *S. pneumoniae* biofilms from amoxicillin treatment in chinchillas' middle ear in OM models (Short et al., 2021).

NTHi biofilms in COPD lung are currently being studied, but most available information on the biofilm life of these bacteria is given through OM studies or *in vitro* bacterial monoculture models (Weeks et al., 2021). NTHi biofilms exhibit a series of mechanisms of resistance and tolerance to several commonly used antibiotics (Table 2). For example, in OM, the antibiotics ciprofloxacin, azithromycin, and amoxicillin, which are clinically effective for planktonic states of NTHi, are ineffective for NTHi biofilms. In addition, the effect of the antibiotics chlorhexidine, glucuronate, ampicillin, and ciprofloxacin is also ineffective due to the rich eDNA matrix (Weeks et al., 2021). A recent study with a COPD model of ferret lung exposed to cigarette smoke showed that NTHi bacteria have the ability to aggregate and express genes related to growth, QS, and environmental and oxidative stress tolerance, which induces formation and persistence of NTHi biofilms (Hunt et al., 2020). Thus, with the increase in AMR, it is necessary to find new ways to combat these versatile and adaptable microorganisms. Therefore, it is essential to study and explore the mechanisms of bacterial resistance, characterize the biofilm formation capacity of NTHi, identify the host-pathogen interactions in COPD, as well as anti-biofilm therapeutic targets. These strategies can have a positive impact on diagnosis and treatment, not only in COPD, but also in terms of other diseases, with the ultimate goal of minimizing this greatest source of mortality and morbidity (C Reygaert, 2018; Weeks et al., 2021).

Table 2 - Summary of NTHi biofilm resistance and tolerance mechanisms and their respective model and experimental effects (adapted from Weeks et al., 2021).

Mechanism and Drug	Model/Experiment	Experimental effects	Citation
Gentamycin tolerance	Clinical CF[1] NTHi isolates adhered to human airway epithelia	Biofilms survived treatment with high gentamycin concentration.	(Starner et al., 2006)
β-lactam antibiotics & carbohydrate metabolism	NTHi biofilms grown on airway epithelia	Increase in carbohydrate metabolism gene expression in response to sub-MIC ampicillin and amoxicillin.	(Wu et al., 2014)
eDNA-rich EPS matrix protects against antimicrobials	8 clinical NTHi isolates static biofilms *in vitro*	Biocide resistance mediated by the cohesive and protective properties of the biofilm matrix.	(Izano et al., 2009)
Low metabolic activity protects biofilms against β-lactam antibiotics	Metabolomic and proteomic analysis of 814 proteins across biofilm and planktonic strains.	127 differentially expressed products suggested that NTHi biofilms survive β-lactam antibiotics in a dormant state with decreased energy metabolism and protein synthesis.	(Post et al., 2014)

2.7 AMPs against bacteria

Bacterial resistance to antibiotics, which are one of the main ways of treating infections, is a major concern in modern medicine. Currently, the slow pace of discovery and development of new antibiotics in the face of the rapid spread of resistance by these microorganisms to these drugs causes serious impacts on the economy and public health (Aslam et al., 2018; Hernández-Aristizábal & Ocampo-Ibáñez, 2021). Therefore, it is urgent to find new antimicrobial drugs against bacteria.

AMPs are multifunctional molecules of the innate immune system of prokaryotic and eukaryotic organisms that act against infections caused by bacteria, fungi, viruses, and some protozoa (Hernández-Aristizábal & Ocampo-Ibáñez, 2021; Wu et al., 2018). Generally, in nature, the different types of AMPs are constituted by four, six, or eight cysteines interconnected by disulfide bridges that confer stability, have between 12 and 50 amino acids, α-helical structure, are mostly cationic, and show amphiphilicity (Denardi et al., 2022; Hernández-Aristizábal & Ocampo-Ibáñez, 2021; H. Wang et al., 2022). The α-

[1] CF – Cystic Fibrosis

helical structure and positive charges are important for the bioactivity of these peptides (H. Wang et al., 2022).

Their unique characteristics confer broad antibacterial properties, presenting a series of mechanisms that act in the disruption of the cell membrane or alteration of the permeability (membrane targeting action) (P. Kumar et al., 2018; R. Kumar et al., 2020). However, there are other mechanisms of AMPs that act at the intracellular level (non-membrane action). Interaction with the cell membrane is the initial step in the mechanisms of action of AMPs (Bahar & Ren, 2013). These, due to their positive charge and hydrophobicity, through electrostatic interactions, can combine with negatively charged structures on the surface of bacterial lipid membranes that lead to their penetration into the phospholipid bilayer. At this stage, the amphipathic structure of AMPs interacts with the hydrophobic regions and, therefore, their degree of hydrophobicity is essential for permeability in the phospholipid bilayer (Ma et al., 2020; H. Wang et al., 2022).

The mechanisms of disruption of the cytoplasmic membrane of AMPs are performed through the interaction of AMPs with negative charges on the cytoplasmic membrane, which consequently increase membrane permeability, cause cell membrane lysis or release of intracellular components that lead to bacterial death. Some models have been proposed to explain the action of AMPs on cytoplasmic membrane disruption, such as barrel-stave model, toroidal-pore model, carpet model, and aggregate model. In these models, membrane rupture occurs when AMP penetrates the phospholipid bilayer and its hydrophobic regions combine with the hydrophobic regions inside the phospholipid bilayer, leaving the hydrophilic regions facing outwards (Zhang et al., 2021). As mentioned above, AMPs can still kill bacteria without causing membrane permeabilization. These are called intracellularly active AMPs, where the bactericidal effect begins with the interaction of AMP with the cytoplasmic membrane and then its accumulation in the intracellular environment, which can inhibit critical cellular functions leading to the death of the bacterium (Bahar & Ren, 2013; P. Kumar et al., 2018). Inside the bacterial cell, they can thus inhibit cell wall synthesis through the interaction of precursor molecules that are essential for cell wall synthesis, as well as inhibit the synthesis of nucleic acids (DNA and RNA) and proteins (P. Kumar et al., 2018). AMPs with intracellular action also have the ability to inhibit enzymatic action, such as the action of proteases, and can promote the release of cell wall lyases that hydrolyze cell structures, which can result in cell lysis (Zhang et al., 2021).

AMPs are considered new alternatives to traditional antibiotics because they have a high potential to prevent bacterial resistance, as they quickly kill bacteria and, unlike antibiotics, which target pathogen-specific molecular receptors, AMPs have a different mechanism of action and may act on multiple targets

on the cytoplasmic membrane and on intracellular targets of pathogenic bacteria (J. Wang et al., 2019; Zhang et al., 2021). The combination of AMPs with antibiotics can increase microbial inhibitory activity. This synergism can, for example, facilitate the entry of the antibiotic into the bacteria due to the increase in the permeability of the bacterial membrane by AMPs and can also inhibit the efflux pumps preventing the expulsion of the antibiotics (Baltzer & Brown, 2011). Furthermore, the lowest effective dose of the antibiotic and AMP needed to kill the bacteria dramatically slows the development of resistance and toxicity (Hao et al., 2022). Thus, AMPs increase the lifespan of antibiotics, and may be potential adjuvants of antibiotics for the treatment of infections (Li et al., 2020; Mishra et al., 2017). AMPs also have antibiofilm activity against Gram-positive and Gram-negative bacteria, for example, through interference with bacterial signaling molecules, as well as interference with regulatory pathways that lead to the persistent phenotype (Batoni et al., 2016). Furthermore, AMPs that target the bacterial membrane can efficiently kill dormant or non-growing cells, such as persistent cells, since their activity does not require the cells to be metabolically active (Jorge et al., 2019). Their prevalent and different mode of action from conventional drugs on bacterial membranes makes them effective against multidrug-resistant bacteria. AMPs can also negatively regulate extracellular matrix biosynthesis, potentiate the activities of host immune cells through their recruitment to the site of infection, and also promote healing processes by stimulating cell proliferation or angiogenesis, which contributes to tissue repair during the course of biofilm infections (Batoni et al., 2016).

There are some disadvantages of AMPs, such as the development of resistance by some bacteria to AMPs, although this is not easily inducible due to the physical disruption of the bacterial membrane and intracellular targets. The mechanisms of bacterial resistance to AMPs include efflux pumps, transporters of uptake and proteolytic degradation, as well as cell surface modification, such as the reduction of negative charges on the membrane (J. Wang et al., 2019). Even though AMPs have potential for treating infections, the Food and Drug Administration has only authorized a small number of peptides (FDA). Most clinical assays are limited to topical assays due to systemic toxicity, blood cytotoxicity, susceptibility of peptides to proteolytic degradation, and rapid renal clearance if ingested. To circumvent these problems and improve the effectiveness of AMPs, chemical modification is being implemented to promote activity and biocompatibility, as well as the design of new AMPs and AMP delivery systems, helping to improve stability, toxicity, shelf-life and their release profile (P. Kumar et al., 2018).

Chapter 3. Materials and Methods

Chapter 3 refers the bacterial strains used, their storage conditions, and the reagents used, and describes the laboratory methodologies and the main material needed to carry out the experiments.

3.1 Bacterial Strains and Cell Storage

In the present study, the reference strains *H. influenzae* DSM 4690 (ATCC 33391, NCTC 8143) and *S. aureus* (ATCC 25923) were used. All bacteria were stored at -80 °C ± 2 °C in preservation tubes (cryovials) with their respective growth media supplemented with 20% glycerol.

3.2 Growth Media and Initial Culturing

Brain Heart Infusion (BHI) broth from three brands, namely VWR, Liofilchem, and Oxoid, was used in this work. The BHI was prepared according to the supplier instructions and supplemented with 10 µg/mL of NAD (ACROS organics) and 10 µg/mL of Hemin (Alfa Aesar) immediately before use. Supplements were prepared as follows: 10 mg/mL of NAD was dissolved in distilled water and stored at -20 °C, while 10 mg/mL of Hemin was dissolved in 0.1M NaOH (Sigma-Aldrich®) and stored at 4 °C. Supplemented Brain Heart Infusion Broth (sBHI) was used for the liquid cultivation of *H. influenzae* and *S. aureus*.

For the cultivation in solid media, 15 g/L of agar was added to the BHI broth and, after autoclaving, it was supplemented with the same concentrations of NAD and Hemin. The supplemented Brain Heart Infusion Agar (sBHIA) plates were stored at 4 °C up until use.

In the initial culturing, *H. influenzae* preserved in the cryovials was streaked weekly and *S. aureus* monthly and grown in sBHIA for 24 h at 37 °C with 5% CO_2. Then, *H. influenzae* colonies were stored at room temperature in a candle jar for a maximum of one week, and *S. aureus* colonies were stored at 4 °C and re-streaked after one week.

3.3 Biofilm Formation

For biofilm formation, each strain was inoculated in 15 mL of sBHI and incubated overnight (37 °C, 120 rpm, 5% CO_2), centrifuged (9000 × g, room temperature, 5 min) and re-suspended in sBHI until reaching 1 x 10^6 CFU/mL. The optical density (OD) value (at 620 nm) of the cell suspension and the equation of the calibration curve of each strain (Table 3), previously created by the research group, were used to determine this concentration. Mixed bacterial suspensions comprised of both species were achieved by mixing different proportions of each mono-species suspension, therefore maintaining the

same total cell concentration of 1 x 10^6 CFU/mL. A total of 200 µL of the prepared bacterial suspensions (single or dual-species) were transferred to each well of a flat-bottom 96-well polystyrene microtiter plate (Orange Scientific) and incubated for 24 h or 48 h (37 °C, 120 rpm, 5% CO_2). In some instances, the growth media was replaced at 24 h for the 48 h biofilms, in which case the sBHI with cells still in suspension was removed and fresh sBHI was gently added to not disturb the adhered cells.

Table 3 - Calibration curve equations for the two bacterial strains provided by the research group.

Strains	Calibration curve equations
H. influenzae DSM 4690 (ATCC 33391, NCTC 8143)	$Concentration\ (CFU/mL) = 1.34 \times 10^8\ \times OD - 1.27 \times 10^7$
S. aureus (ATCC 25923)	$Concentration\ (CFU/mL) = 9.8 \times 10^8\ \times OD + 7 \times 10^7$

3.4 Quantification of Culturable Bacterial Cells in Biofilms

After biofilm growth, the media containing non-attached cells was removed and the wells were washed twice with BHI to remove remainder planktonic cells from the biofilms. Then, 200 µL of BHI was added and the walls and bottom of the wells were scraped in order to promote the detachment of the biofilm cells. The resulting cell suspensions were collected and vortexed for approximately 40 s and serial dilutions (1:10) were made in BHI broth. For the analysis of double-species biofilms, the bacterial suspensions were plated in specific selective solid media. For the growth of *S. aureus,* the selective medium used was Mannitol Salt Agar (MSA) (Liofilchem), and, for the growth of *H. influenzae*, the selective medium was sBHIA supplemented with the antibiotics clindamycin (1 µg/mL) (Sigma), vancomycin (5 µg/mL) (Applichem), and bacitracin (300 µg/mL) (PAN BioTech). All antibiotic stocks, namely 2 mg/mL of clindamycin and 10 mg/mL of vancomycin, were prepared in sterile distilled water and stored at -20 °C. Bacitracin was weighted and dissolved in 1 mL sterile distilled water immediately prior to use. The plates of *H. influenzae* and *S. aureus* were incubated (37 °C, 120 rpm, 5% of CO_2) for 48 h and 24 h, respectively. Finally, the concentration of culturable biofilm cells was determined by CFU counting and expressed as Log_{10} (CFU/cm^2), taking into account the volume of suspension added to the well of 200 µL, the submerged well surface area of 1.53 cm^2, and the plated volume of 10 µL.

3.5 Biofilm Biomass Quantification by Crystal Violet (CV) Staining

The chemical method of CV staining, adapted from Stepanović and colleagues, was performed to quantify the total biomass of biofilms (Stepanović et al., 2000). After washing the biofilms, as previously

described, they were fixed with 250 µL of pure methanol (Romil) for 15 min. Subsequently, the methanol was removed by inversion of the plates, which were then air dried, and the fixed biofilms were stained with 250 µL of CV at 1% (V/V) (Merck) for 10 min. Then, excess CV was removed from the plates by inversion and washing the wells (twice) with 250 µL of distilled water. The wells of the plates were again emptied by inversion, air dried, and the remaining CV bound to the fixed biofilm was solubilized with 200 µL of 33% (V/V) acetic acid (Fischer Scientific). Finally, the OD_{595} of the solutions was measured.

3.6 Influence of Growth Media Brand in *H. influenzae* Biofilm Formation

To test if the brand of growth media altered the formation of *H. influenzae* biofilms, 24 h and 48 h biofilms were formed (section 3.3) in sBHI of three different brands, namely VWR, Liofilchem, and Oxoid. In the end, biofilm cells were evaluated for their culturability through the CFU method (section 3.4), and the amount of biomass present was quantified using the CV method (section 3.5).

3.7 Population Dynamics in Dual-species Biofilms

To investigate population dynamics between *S. aureus* and *H. influenzae*, 24 h and 48 h dual-species biofilms of *H. influenzae* were formed (section 3.3) with different initial concentrations of *S. aureus* (1%, 10%, 25%, and 50% V/V). Single *H. influenzae* and *S. aureus* biofilms were performed as controls for this procedure. Finally, the culturable cells were determined by the CFU method (section 3.4) as well as the amount of biomass by the CV method (section 3.5).

3.8 Influence of the Stepwise Colonization on Biofilm Formation

To study the influence of sequential colonization by different species, biofilms of *H. influenzae* and *S. aureus* were formed (section 3.3). After 24 h, the content of each well was removed apart from the adhered biofilm and a bacterial solution of the other species at 1 x 10^6 CFU/mL was added. The biofilms were grown for an extra 24 h. Controls corresponding to the single 24 h and 48 h biofilms of *H. influenzae* and *S. aureus* were performed. In the 48 h biofilm controls, sBHI medium was exchanged at 24 h. Biofilms were analyzed through culturable cell (section 3.4) and biomass quantification (section 3.5).

3.9 Interspecies Influence of Exoproducts on Biofilm Formation

To determine the effect of *S. aureus* and *H. influenzae* exoproducts on the biofilm formation of the other species, single species biofilms were grown for 24 h (section 3.3). After 24 h, the cells were separated by centrifugation (3134 x g, room temperature, 5 min), and the supernatant, which would

contain any existing soluble exoproducts, was filtered through a 0.22 µm low binding filter (FilterBio) for sterilization. Then, biofilms of each mono-species were washed once with BHI medium to remove planktonic cells. The cell-free supernatant (CFS) of *S. aureus* and *H. influenzae* was added to the biofilm of *H. influenzae* and *S. aureus*, respectively, as well as to biofilms of the same species as a control. CFS sterility was confirmed by plating it onto a sBHIA plate. The influence of exoproducts was analyzed through cell culturability by CFU counting (section 3.4).

3.10 AMPs Susceptibility Testing

The broth microdilution method was used to test the antimicrobial susceptibility of *H. influenzae* and *S. aureus* to the AMPs temporin A, tachyplesin I, palm-KGK-PEG, and citropin 1.1. The effect of AMPs was determined by the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Mueller-Hinton broth supplemented with 5% lysed horse blood and 20mg/L β-NAD (MH-F broth) was used for testing *H. influenzae,* and un-supplemented cation-adjusted Mueller-Hinton broth (MHB) was used for testing *S. aureus*, since it is a non-fastidious bacterium (EUCAST, 2022c). Initially, 100 µL of several concentrations of AMPs (1 mg/L to 1024 mg/L) were prepared in the respective growth media for each bacterium. Then, 100 µL of bacterial suspensions with a concentration of 1 x 10^6 CFU/mL were added to each well with antimicrobial solution, making a total volume of 200 µL and a final cell concentration of 5 x 10^5 CFU/mL. Finally, the plates were incubated at 37 °C, 120 rpm, with 5% CO_2, for 24 h. In this method, three controls were performed: a negative control (growth of bacteria without any antibiotic), a positive control (growth of bacteria with ciprofloxacin antibiotic), and a media sterility control (only growth media, no bacteria). Following the European Committee on Antimicrobial Susceptibility (EUCAST) guidelines, the MIC was determined by the first well without turbidity and pellet in the bottom of the well. For the MBC, 10 µL from each well without visible bacterial growth was plated in sBHIA and, after incubation (24 h, at 37 °C, with 5% CO2), the MBC values were defined as the lowest concentrations capable of inhibiting colony growth. All assays of each condition were performed in triplicate (EUCAST, 2022a).

3.11 Statistical Analysis

Between one and five independent assays were performed for each experiment with three or more replicates per condition. The graphs and statistical tests were executed in GraphPad Prism (version 9, GraphPad Software, San Diego California USA). All results presented in graphs include means ± standard deviations (SD). The statistical tests performed were the unpaired T-test, as well as the ordinary one-way

ANOVA and two-way ANOVA using Bonferroni's multicomparison test. The significant differences between the groups are represented in the graphs by the asterisk (*) or the cardinal (#) symbols, where */# equals $p < 0.05$, **/## equals $p < 0.001$, ***/### equals $p < 0.001$, and ****/#### equals $p < 0.0001$.

Chapter 4. Results and Discussion

Chapter 4 is composed of the description and depiction of the results obtained, their critical analysis as well as their discussion.

4.1 Impact of media renewal on the growth of *H. influenzae* biofilm

To study the impact of growth media renewal in the biofilm formation of *H. influenzae*, 48 h biofilms were formed, with and without media replacement at 24 h. Based on the analysis of Figure 4, it is possible to verify that, when there is no replacement of media at 24 h, there are no culturable bacteria at 48 h. On the other hand, when the medium is replenished at 24 h, there are culturable bacteria at 48 h. The results suggest that sBHI medium replacement affects the amount of culturable bacteria present in the biofilm, since there are statistically significant differences between *H. influenzae* counts without sBHI medium replacement compared to *H. influenzae* counts with sBHI medium replacement ($p < 0.0001$) (Figure 4). These results are in agreement with those previously obtained by our research group (data not published).

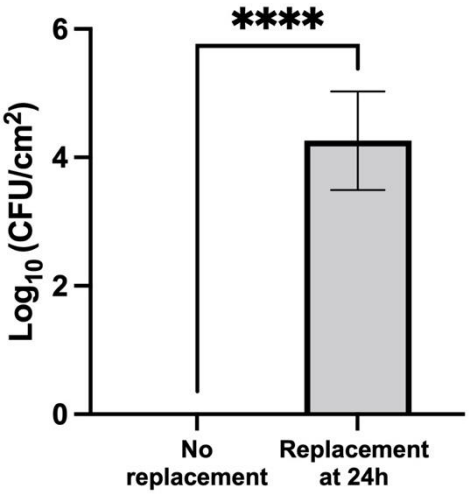

Figure 4 - Quantification of culturable *H. influenzae* in 48 h biofilms grown in sBHI (Liofilchem), with and without media replacement at 24 h. On the graph, standard deviations are indicated by error bars. Statistically significant differences: **** p<0.0001.

The development of biofilms and their bacterial and final chemical composition are affected by the amount, availability, and type of nutrients and by their perfusion to the cells inside of the biofilm (Bowden & Li, 1997; Dunne, 2002). Hence, the absence of culturable cells observed could be explained by bacterial unviability caused by consumption and consequent depletion of nutrients by the bacteria over time, such as the growth factors hemin and NAD. *H. influenzae* cells are not well adapted to survival under non-optimal conditions, requiring an enriched culture medium, namely BHI, with adequate supplementation of hemin and NAD (Poje & Redfield, 2003).

The toxic metabolites produced by the bacteria themselves are also another factor that could explain the absence of culturable bacteria. Dunne describes that, in bacterial biofilms, bacterial growth is limited by the expression of quorum-sensing molecules released in response to nutrient limitation and the accumulation of toxic by-products (Dunne, 2002). *H. influenzae* is subject to oxidative stress resulting from its own metabolism during aerobic growth (Harrison et al., 2012). Oxidative stress can cause damage to all types of bacterial cell components including proteins, lipids, and DNA, leading to bacterial death (Ezraty et al., 2017; Fasnacht & Polacek, 2021).

A third hypothesis to explain the absence of culturable cells is that *H. influenzae* could be viable but not culturable (VBNC). In the VBNC state, the biofilm bacteria are alive but do not grow or divide, and their presence is not detectable by conventional methods (they do not form colonies on solid media and do not change the appearance of the broth) (Năşcuţiu, 2010). The VBNC state is a unique survival strategy in the face of adverse environmental conditions such as antibiotic pressure, high or low temperature, starvation, chlorination, pH change, and oxygen stress (Ramamurthy et al., 2014). It is known that VBNC has been associated with longer periods of biofilm formation and, furthermore, the central areas of the same tend to be hypoxic, with limited resources, and acidic due to the deposits of metabolic residues that consequently induce the VBNC state (Ayrapetyan et al., 2018; Castro et al., 2022). The VBNC state can be reversible under favorable growth conditions with an ideal energy source and stoichiometric ratio of carbon to inorganic elements (Ramamurthy et al., 2014).

4.2 Influence of Growth Media Brand in *H. influenzae* Biofilm Formation

Previous assays within the research group found behavioral variations in the growth of *H. influenzae*. The assays were highly consistent for about 5 months, but variability started to be more worrisome afterwards (data not published). Based on Chapter 3 of the book "*Haemophilus influenzae* protocols", growing *H. influenzae* on cheaper growth media can cause these growth problems as well as plating problems (Poje & Redfield, 2003). As such, the influence of media brand on *H. influenzae* biofilm growth was tested.

4.2.1 *H. influenzae* Cryovial Variability in Different Brands of Growth Media

Surprisingly, the variability detected in the growth of *H. influenzae* by the research group was suspected to correlate with different cryovials of the same strain. Although all the vials were prepared and stocked at the same time, repeated use of some could be compromising the cells. For this reason, the differential growth between two cryovials was tested, one of which was already in use and the other was opened for the first time. In addition, it was also investigated whether the variability in the growth of these

cryovials was dependent or not on the brand of growth media used, namely, Oxoid (most expensive), VWR (average price), and Liofilchem (cheaper and used in all previous studies within the group).

For this study, 24 h and 48 h biofilms of *H. influenzae* (with and without media replacement) were formed in sBHI of different brands. The sBHI from VWR was discarded at the beginning of the assay because HI 2 (*H. influenzae* from cryovial 2, in use) did not grow in it, hence no comparison was performed for this brand. However, this was in itself an interesting result, since it was possible to see that there is in fact differences in bacterial growth dependent on the media brand and the cryovial used.

4.2.1.1 24 h Biofilms

Figure 5 illustrates the results of culturable cells and total biomass obtained in the cultivation of 24 h *H. influenzae* biofilms. It is possible to observe that, in sBHI from Oxoid, the number of culturable cells (Figure 5-A) as well as the amount of total biomass of the biofilm (Figure 5-B) is similar for the two cryovials. In contrast, with the sBHI from Liofilchem, HI 2 has fewer culturable cells (1 log) ($p < 0.01$) (Figure 5-A) and drastically lower biomass ($p < 0.0001$) (Figure 5-B) compared to HI 6 (*H. influenzae* from cryovial 6, newly opened). In addition, it is observed that the Oxoid brand reached a higher number of culturable cells compared to the Liofilchem brand (Figure 5-A) and, in terms of biomass (Figure 5-B), sBHI from Oxoid performed equal for HI 6 and better for HI 2.

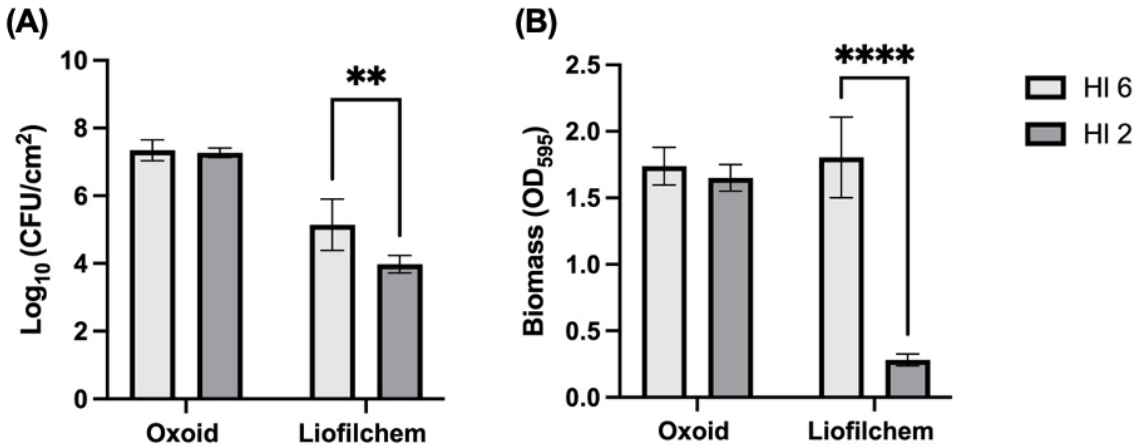

Figure 5 - Quantification of culturable *H. influenzae* cells (A) and total biomass (B) of 24 h *H. influenzae* biofilms grown in sBHI from Oxoid and Liofilchem. Standard deviations are indicated by error bars. Statistically significancy: ** $p < 0.001$ and **** $p < 0.0001$. Abbreviations: HI 2 = *H. influenzae* from the second cryovial (in use), HI 6 = *H. influenzae* from the sixth cryovial (newly opened).

The growth differences observed suggest that the proliferation of *H. influenzae* may be dependent on the brand of sBHI used, which could be related to its higher or lower quality. The results also showed that the amount of biomass (Figure 5-B) does not directly correlate with the amount of culturable cells, since HI 6 produced a similar amount of biomass in both brands but had a lower amount of culturable

cells when grown in sBHI from Liofilchem (Figure 5-A). This similarity in the amount of biomass in both brands can be explained by the CV method which, in addition to quantifying viable cells, also quantifies dead cells and extracellular matrix (Xu et al., 2016). Interestingly, the amount of biomass differed significantly for the Liofilchem brand. This phenomenon can be explained by the greater production of extracellular matrix of HI 6 compared to HI 2, even though they are of the same strain. This could indicate that HI 2 suffered modifications derived from its use that caused cells to lower their ability to produce matrix.

Additionally, it was verified that biofilms with 24 h have culturable cells of *H. influenzae*. Thus, the problems mentioned in the previous section (4.1), such as lack of nutrients and production of toxic metabolites by *H. influenzae* that can affect the culturability of the biofilm, are not yet present in biofilms cultured for 24 h.

4.2.1.2 48 h Biofilms without Media Renewal

Figure 6 shows the same comparison as before but for 48 h biofilms without media renewal at 24 h. Compared to 24 h *H. influenzae* biofilms, the amount of culturable cells (Figure 5-A) reduced for all conditions, except for HI 2 with the sBHI from Liofilchem (Figure 6-A). This could indicate that the cell growth of vial HI 2 was slower than that of vial HI 6 in Liofilchem and, therefore, they managed to grow a little more from 24 h to 48 h, as they could still be at the end of the exponential phase or in the stationary phase. In the other three conditions, as the cells could have grown faster, they could have already reached the dead phase or in the biofilm detachment stage, resulting in lower cell culturability at 48 h. For sBHI from Oxoid, the amount of culturable HI 2 cells was 2 logs lower compared to HI 6 ($p < 0.05$) (Figure 6-A) but the amount of biomass was identical for both cryovials (Figure 6-B). Furthermore, Figure 6-A shows that there is a significant difference ($p < 0.01$) in the number of culturable cells between HI 2 and HI 6 for sBHI from Liofilchem. This can be explained by the same reason described above. In terms of biomass, there is a significant reduction ($p < 0.0001$) between the two cryovials (Figure 6-B) that was also observed in the 24 h *H. influenzae* biofilms (Figure 5-B) and discussed above. It is noteworthy that the amount of culturable cells and biomass differs between brands and cryovials, except for the amount of biomass in cryovials of sBHI from Oxoid (Figure 6-B).

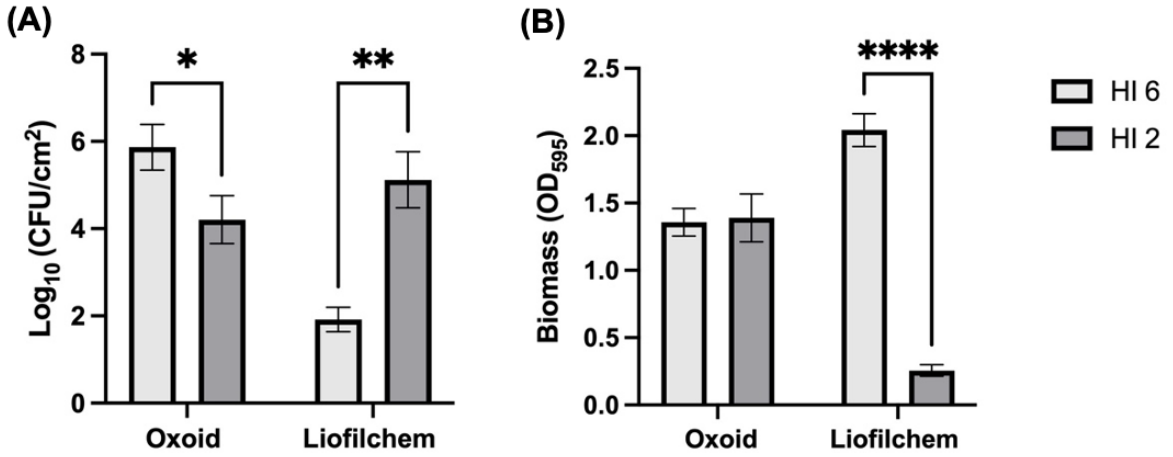

Figure 6 - Quantification of culturable *H. influenzae* (A) and total biomass (B) of 48 h *H. influenzae* biofilms grown in sBHI from Oxoid and Liofilchem without media replacement at 24h. Standard deviations are indicated by error bars. Statistically significancy: * $p < 0.05$ ** $p < 0.01$, and **** $p < 0.0001$. Abbreviations: HI 2 = *H. influenzae* from the second cryovial (in use), HI 6 = *H. influenzae* from the sixth cryovial (newly opened).

Interestingly, culturable cells of these 48 h biofilms without media renewal were observed for both brands (Figure 6-A), contrary to the initial experience (Figure 4), which was performed with sBHI from Liofilchem. This confirmed the variability previously observed by the research group and eliminated the operator as the possible causing factor. This event can be related to the universal stress protein (UspA). Although lack of sBHI replacement is associated with loss of culturability probably due to deficiency of nutrients and production of toxic metabolites, as discussed previously, it is known that *H. influenzae* can survive due to increased expression of UspA. According to the literature, when bacteria are exposed to stressful environmental conditions that compromise cell viability, UspA expression is increased, enhancing the bacterial survival rate (Sousa & McKay, 2001). In this case, the probable stress caused by nutrient depletion and production of reactive oxygen species resulting from its own metabolism, which could compromise cell viability, may have induced an increase in UspA expression.

4.2.1.3 48 h Biofilms with Media Renewal

Finally, Figure 7 shows the same comparisons as before but for 48 h biofilms with media renewal at 24 h. It is possible to observe that the amount of culturable *H. influenzae* cells for sBHI from Oxoid was significantly higher ($p < 0.001$) (Figure 7-A) in vial HI 2 than in vial HI 6. Regarding total biomass, there was similar production for both vials (Figure 7-B). The amount of culturable *H. influenzae* cells for sBHI from Liofilchem was also higher ($p < 0.001$) (Figure 7-A) in HI 2 than in HI 6. In contrast, the amount of biomass produced was significantly lower ($p < 0.0001$) (Figure 7-B) in HI 2 compared to HI 6. This difference in the quantification of biomass between cryovials for sBHI from Liofilchem ($p < 0.0001$) (Figure 7-B) has also been observed previously (Figure 5-B and 6-B).

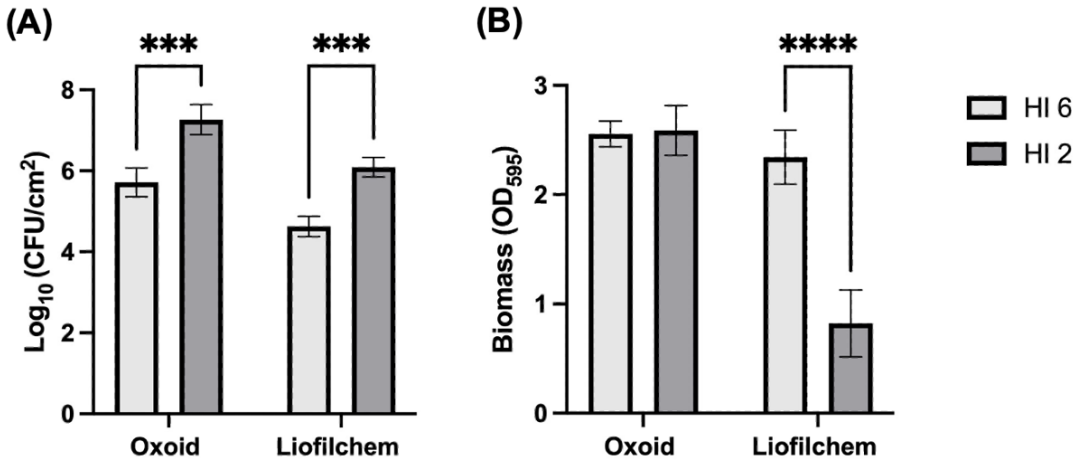

Figure 7 – Quantification of culturable *H. influenzae* (A) and total biomass (B) of 48 h *H. influenzae* biofilms grown in sBHI from Oxoid and Liofilchem with media replacement at 24 h. Standard deviations are indicated by the error bars. Statistically significancy: *** $p<0.001$ and **** $p<0.0001$. Abbreviations: HI 2 = *H. influenzae* from the second cryovial (in use), HI 6 = *H. influenzae* from the sixth cryovial (newly opened).

Comparing the results of 48 h biofilms without (Figure 6) and with media renewal (Figure 7), it is possible to verify an increase in the number of culturable cells for both cryovials (Figure 7-A) and total biomass (Figure 7-B) for sBHI from Oxoid. As verified in the initial experiment (Figure 4), the replacement of sBHI at 24 h showed again that the restitution of nutrients and the possible removal of toxic metabolites could be what is here influencing cell culturability.

In conclusion, it was found that there were behavioral differences between HI 2 and HI 6 cryovials, even though they were from the same strain and established at the same time. Thus, it was seen that vial HI 2, which had already been opened and consequently subjected to more temperature variations, due to its freezing and thawing, had a slower bacterial growth compared to the vial HI 6, which was opened for the first time to be used in this experiment. Furthermore, *H. influenzae* growth differences were dependent on the brand of growth medium used, and variability and poorer/slower growth and biomass formation were most noticeable for sBHI from Liofilchem. The results of sBHI from Oxoid were more consistent among the cryovials tested. Thus, the Oxoid brand was chosen to carry on the remainder assays.

4.3 Population Dynamics in Dual-species Biofilms

In biofilms, the interaction of bacteria with each other is inevitable. These interactions can be cooperative or competitive, and can influence the growth and survival of species, having a spatial and temporal impact on the formation of a highly organized community (Giaouris et al., 2015). Throughout the development of the biofilm, microorganisms are distributed in a non-random manner depending on

social interactions and nutrient availability (W. Liu et al., 2018; Paula et al., 2020). In a multispecies biofilm, there are interactions that benefit the whole group, such as the availability of synthesized compounds and the escape of host defenses, but there are also harmful interactions, such as species elimination due to competition from different coexisting species (Reigada et al., 2021). Investigation of population dynamics is essential for understanding interactions between species, making possible the selection of drugs that can inhibit interactions between species and their ability to form multispecies biofilms (Tikhomirova & Kidd, 2013a).

4.3.1 Population Dynamics of Dual-Species Biofilms in Growth Media of Different Brands

Given the variability observed previously regarding the use of different media brands in the growth of *H. influenzae* biofilms, a preliminary assay was performed to check if the media brand had the same effects when *S. aureus* was mixed with *H. influenzae*. In this way, 24 h and 48 h *H. influenzae* biofilms (single-species control) and *H. influenzae* biofilms with 1% of *S. aureus* (starting inoculum %) were formed in sBHI from Liofilchem and from Oxoid.

4.3.1.1 24 h Biofilms

The results of 24 h *H. influenzae* biofilms grown in sBHI from Liofilchem show statistically significantly lower culturable cells when compared to Oxoid ($p < 0.0001$) (Figure 8-A), in contrast to the amount of biomass, which is similar) (Figure 8-B). This fact occurs for the same reasons explained in the previous study, namely the greater probability of sBHI from Liofilchem to cause growth problems. In terms of total biomass, although equal values were obtained, biofilm composition may be different, as it may contain a greater amount of dead cells for sBHI from Liofilchem due to the lower culturable cells counts. The results of *H. influenzae* biofilms grown with 1% S. aureus showed no significant difference in the number of culturable cells (Figure 8-A) or biomass (Figure 8-B) between the two brands. Interestingly, the amount of culturable *H. influenzae* cells was higher in the double-species biofilm when compared with the single-species control for sBHI from Liofilchem (Figure 8-A). Thus, *S. aureus* may have a positive influence on *H. influenzae* growth, possibly by providing the necessary nutrients to maintain the culturability of *H. influenzae* cells. In fact, *H. influenzae* and *S. aureus* were already reported to establish a cooperative relationship in polymicrobial infections, in which *H. influenzae* reached a greater number of colonies when *S. aureus* was the resident colonizer due to nutrients that the latter provided, such as hemin and NAD (Nair et al., 2014). In this case, the mechanism for obtaining nutrients by *H. influenzae* was different since sBHI does not have erythrocytes for the hemolysins produced by *S. aureus* to cause

their lysis and release hemin and NAD. In terms of total biomass, this was maintained for all biofilms in the different brands. However, its composition may vary because, for the reasons explained previously.

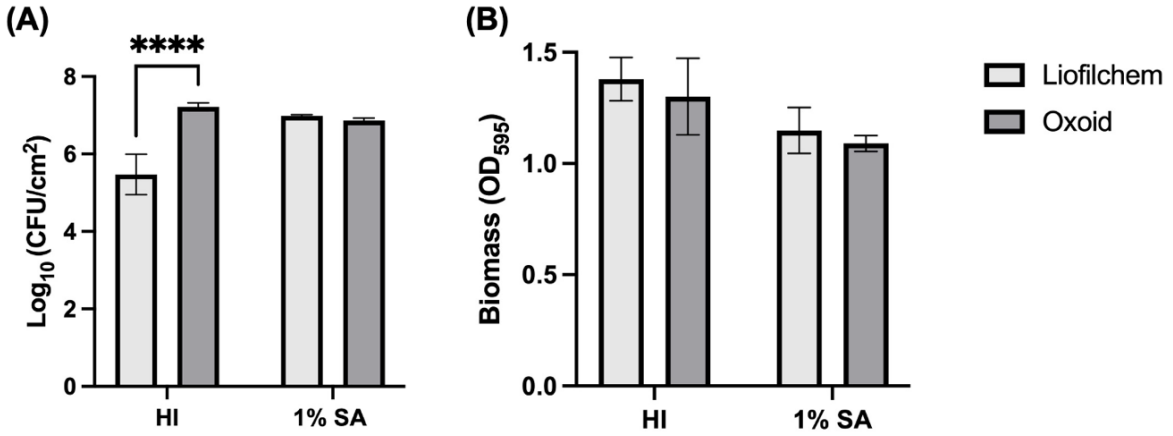

Figure 8 - Quantification of culturable *H. influenzae* (A) and total biofilm biomass (B) from 24 h biofilms of *H. influenzae* (HI) and of *H. influenzae* with 1% of *S. aureus* (1% SA) grown in sBHI from Liofilchem and Oxoid. Statistically significant differences: **** $p < 0.0001$. Standard deviations are indicated by error bars.

4.3.1.2 48 h Biofilms without Media Renewal

In the 48 h *H. influenzae* biofilms without sBHI replacement, a greater number of culturable cells were obtained for sBHI from Oxoid ($p < 0.0001$) (Figure 9-A). As for the quantification of the total biomass, the *H. influenzae* biofilms cultivated in sBHI from Liofilchem reached the highest amount of biomass ($p < 0.0001$) (Figure 9-B), but they were the ones that obtained the lowest number of culturable cells (Figure 9-A), therefore it can be inferred that the amount of biomass comes mainly from dead cells and/or matrix. In biofilms with two species, the number of culturable *H. influenzae* cells was lower for sBHI from Liofilchem ($p < 0.001$) (Figure 9-A), as well as the total biomass ($p < 0.0001$) (Figure 9-B).

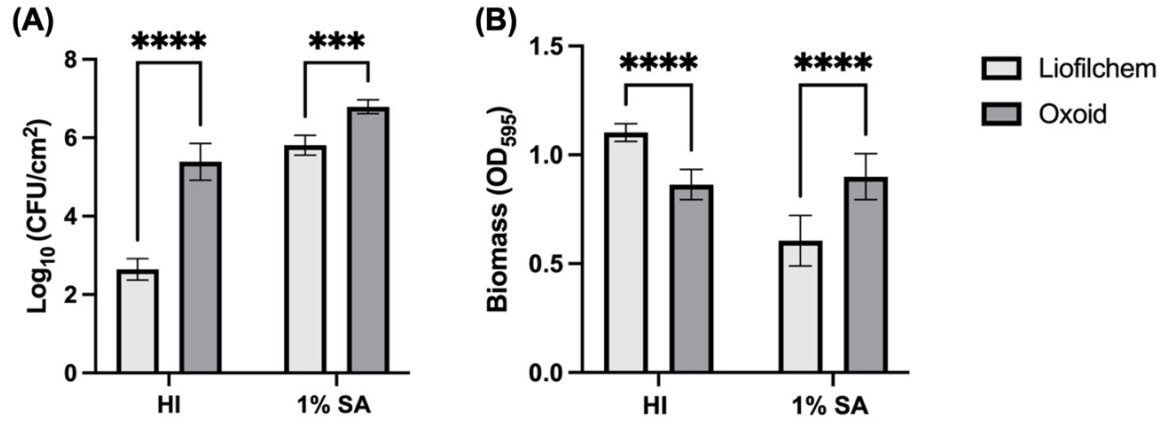

Figure 9 - Quantification of culturable *H. influenzae* (A) and total biofilm biomass (B) from 48 h biofilms of *H. influenzae* (HI) and of *H. influenzae* with 1% of *S. aureus* (1% SA) grown in sBHI from Liofilchem and Oxoid without media replacement at 24 h. Statistically significant differences are represented by *** $p < 0.001$ and **** $p < 0.0001$. Standard deviations are indicated by error bars.

In this case, it is again notorious that the presence of *S. aureus* resulted in a greater amount of culturable *H. influenzae* cells. As described above, *S. aureus* can promote the culturability of *H. influenzae* due to nutrient supply. It is also important to note that when *S. aureus* is present, the amount of biomass is lower in sBHI from Liofilchem compared to the single-species control. This may be due to *S. aureus* making *H. influenzae* produce less matrix, for example.

Comparing *H. influenzae* biofilms at 24 h (Figure 8-A) and at 48 h without media renewal, it was observed that there was a significant decrease in cell culturability, namely for sBHI from Liofilchem (approximately 3 logs) as well as from Oxoid (approximately 1.5 logs) (Figure 9-A). Furthermore, while in the 24 h double-species biofilms (Figure 8-A), the number of culturable cells was similar for the different brands, in this case, the number of culturable cells was lower (approximately 1 log) for Liofilchem (Figure 9-A). Thus, it appears that the lack of sBHI renewal impaired the culturability of *H. influenzae*. In the quantification of biofilm biomass (Figure 9-B), it was shown that, over the 48 h without sBHI replacement, the amount of biomass produced was lower for both biofilms in comparison to the 24 h biofilms (Figure 8-B). Furthermore, the amount of biomass showed more variability between the Liofilchem and Oxoid brands in the 48 h biofilms (Figure 9-B), which did not happen in the 24 h biofilms (Figure 8-B).

Results once again show that the quality of the growth media coupled with the lack of sBHI replacement can influence *H. influenzae* growth. This is mainly shown when the 24 h and 48 h biofilms are compared. For example, when *H. influenzae* biofilms are cultivated in sBHI from Liofilchem, they often reach a lower number of culturable cells in relation to sBHI from Oxoid and, when they are cultivated for a period of 48 h without media replacement, they tend to decrease cell culturability. Interestingly, *S. aureus* positively influenced the amount of culturable *H. influenzae* cells, which leads to the inference that *S. aureus* may play an important role in providing nutrients necessary for the development of *H. influenzae*.

4.3.1.3 48 h Biofilms with Media Renewal

In the 48 h *H. influenzae* biofilms with media renewal at 24 h, the number of culturable cells was higher for sBHI from Oxoid ($p < 0.01$) (Figure 10-A). Regarding the amount of biomass, there was a higher production for sBHI from Liofilchem ($p < 0.0001$) (Figure 10-B) and, as previously explained, it may come from dead cells and/or matrix, since there was less cells cultured. For double-species biofilms, the number of culturable *H. influenzae* cells ($p < 0.01$) (Figure 10-A) and the amount of biomass ($p < 0.001$) (Figure 10-B) was greater for sBHI from Oxoid. Furthermore, it was observed that the *H. influenzae* biofilm had a higher amount of biomass than the double-species biofilm (Figure 10-B).

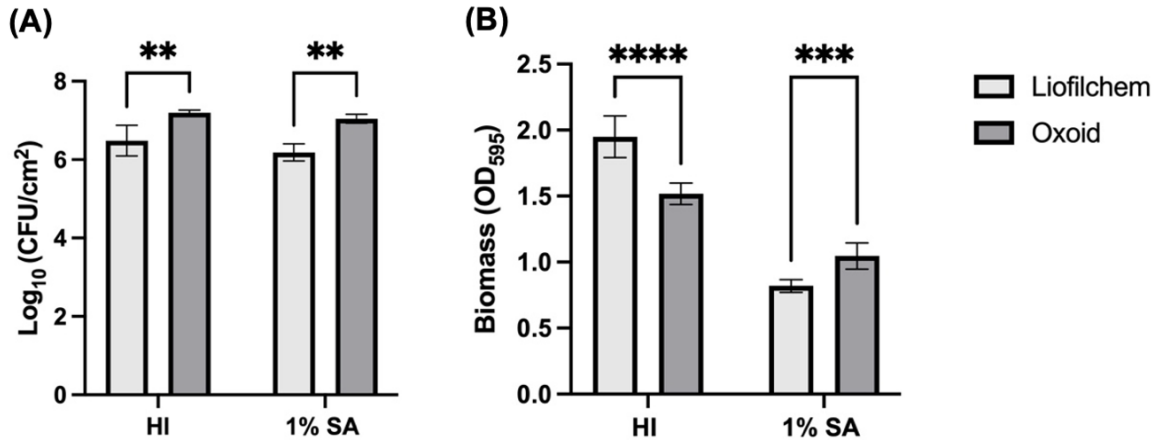

Figure 10 - Quantification of culturable *H. influenzae* (A) and total biofilm biomass (B) from 48 h biofilms of *H. influenzae* (HI) and of *H. influenzae* with 1% of *S. aureus* (1% SA) grown in sBHI from Liofilchem and Oxoid with media replacement at 24 h. Statistically significant differences are represented by ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Standard deviations are indicated by error bars.

When analyzing Figure 10-A, it is mainly noticeable that the number of culturable *H. influenzae* cells increased in *H. influenzae* biofilms in relation to the assay without sBHI replacement (Figure 9-A). This increase may be due to the renewal of sBHI, which added essential supplements for *H. influenzae* survival, thus reaching values closer to double-species biofilms. In double-species biofilms where *S. aureus* may have been providing nutrients, *H. influenzae* maintained culturability. In both biofilms, it was also possible to verify that the Liofilchem brand caused a smaller amount of culturable cells. As previously speculated, this decrease in cell count may be due to the poorer quality of the sBHI from Liofilchem. Analyzing the amount of biomass, it is visible that, in double-species biofilms, the amount of biomass produced was lower than that of biofilms of *H. influenzae*, which could be due to the lower production of extracellular matrix.

In short, as the sBHI from Liofilchem showed greater variability and overall lower biofilm cell counts, it was decided that the sBHI from Oxoid would be used in further studies.

4.3.2 Population Dynamics of *H. influenzae* Biofilms with Increasing Concentrations of *S. aureus*

Population dynamics were accessed for double-species biofilms with different initial concentrations of *S. aureus* (1%, 10%, 25%, and 50% V/V) grown in sBHI from Oxoid. The main objective of this study was to analyze the growth and total biomass produced by *H. influenzae* and *S. aureus* when they grow together in different proportions. Thus, 24 h and 48 h double-species biofilms were formed, where *H. influenzae* and *S. aureus* biofilms were performed as controls (single-species control).

4.3.2.1 24 h Biofilms

Results for 24 h showed that the amount of culturable *H. influenzae* cells for double-species biofilms with 1% and 50% of *S. aureus* was slightly higher compared to the respective single-species biofilm control ($p < 0.05$ and $p < 0.01$, respectively) (Figure 11-A). However, the statistical differences evidenced are not biologically significant given the proximity of the values. Surprisingly, the double-species biofilms, despite having a starting inoculum with low percentages of *S. aureus*, including 1%, reached practically the same number of culturable cells of *S. aureus* in relation to the single-specie biofilm control (Figure 11-A). Thus, it is possible that the relationship between *H. influenzae* and *S. aureus* is mutually beneficial. Not only can *S. aureus* improve *H. influenzae* culturability, as seen previously, but *H. influenzae* could also promote the culturability of *S. aureus*. This type of interaction would allow both bacteria to thrive and establish themselves in the environment in question.

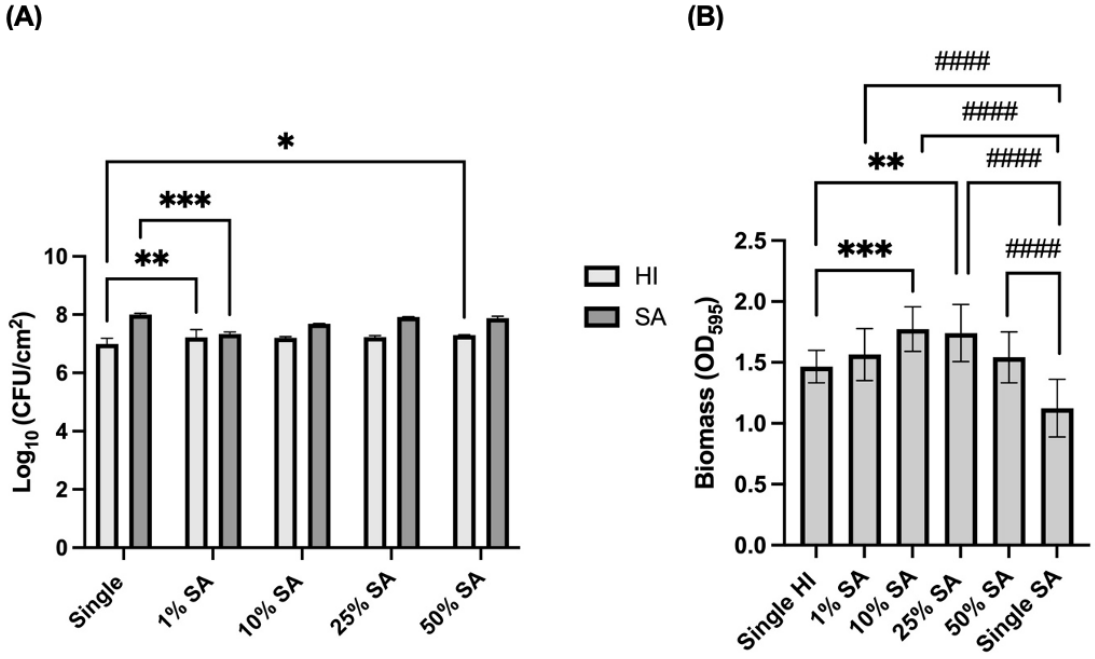

Figure 11 - Quantification of culturable *H. influenzae* and *S. aureus* (A) and total biofilm biomass (B) from 24 h double-species biofilms with different initial concentrations of *S. aureus* (1%, 10%, 25%, and 50% V/V). Statistically significant differences are represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and #### $p < 0.0001$. Standard deviations are indicated by error bars. The symbols * and # represent comparisons with *H. influenzae* and *S. aureus* control biofilms, respectively (single-species control).

As for the total biomass, there was greater production in double-species biofilms with 10% and 25% of *S. aureus* when compared to the *H. influenzae* biofilm control ($p < 0.01$ and $p < 0.001$, respectively) (Figure 11-B). The amount of biomass also increased in all dual-species biofilms compared to the *S. aureus* biofilm control ($p < 0.0001$) (Figure 11-B). Here, there is a tendency for the *S. aureus* control biofilm to produce a lower amount of biomass compared to the other biofilms (Figure 11-B). This happens

because the total biomass of double-species biofilms encompasses cells and matrix from both species, being expected that this value would be equal or higher to the highest single-species biofilms since the species appear to have a positive relationship.

4.3.2.2 48 h Biofilms without Media Renewal

In 48 h dual-species biofilms without sBHI renewal at 24 h, the number of culturable cells was higher in double-species biofilms with 1%, 10%, 25%, and 50% of *S. aureus* compared to the *H. influenzae* biofilm control ($p < 0.0001$) (Figure 12-A). However, the differences in cell counts are not biologically significant given the values proximity. As seen in the previous experiment, even with a reduced initial percentage of *S. aureus*, the amounts of culturable cells were similar to those of controls. This event can be explained by the same reasons described above. Regarding the quantification of biomass, it was higher for biofilms with 1% and 50% of *S. aureus* compared to the control biofilm of *S. aureus* ($p < 0.05$). Additionally, it can also be observed that *H. influenzae* culturability (Figure 12-A) reduced about 10 times when compared to 24 h biofilms (Figure 11-A). As previously mentioned, the depletion of essential nutrients, mainly hemin and NAD for *H. influenzae*, may have influenced this slight decrease in culturable cells in relation to the 24 h biofilms. In terms of biomass quantification, the amount of biomass produced under all conditions (Figure 12-B) was slightly lower than the biomass obtained in the 24 h biofilms (Figure 11-B), as previously verified.

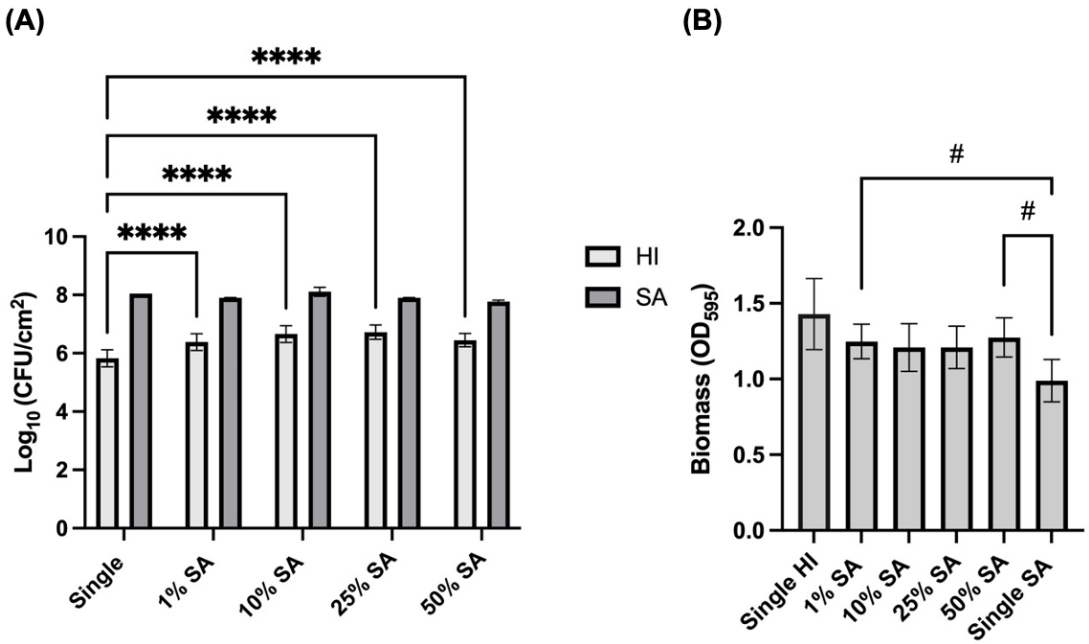

Figure 12 - Quantification of culturable *H. influenzae* and *S. aureus* (A) and total biofilm biomass (B) from 48 h double-species biofilms with different initial concentrations of *S. aureus* (1%, 10%, 25%, and 50% V/V) without media replacement at 24h. Statistically significant are represented by **** $p < 0.0001$, and # $p < 0.05$. The symbols * and # represent comparisons with *H. influenzae* and *S. aureus* control biofilms, respectively.

4.3.2.3 48 h Biofilms with Media Renewal

In 48 h dual-species biofilms with sBHI renewal at 24 h, the number of *H. influenzae* culturable cells was slightly lower in the double-species biofilm with 1% of *S. aureus* when compared to the *H. influenzae* control biofilm ($p < 0.05$) (Figure 13-A). However, at the biological level these differences are not considered significant given the proximity of the values. As in the previous tests, it was possible to verify the double-species biofilms, even with small percentages of *S. aureus*, the number of culturable cells of *S. aureus* was similar to that obtained in the single-specie biofilm control (Figure 13-A). This may indicate that these species can promote cell culturability when living together in a biofilm. In terms of biomass, as described and explained above, the control biofilm of *S. aureus* showed a lower amount of total biomass. Compared with the 48 h biofilms without sBHI replacement (Figure 12-B), the amount of culturable cells (Figure 13-A) and the amount of biomass (Figure 13-B) increased slightly. This increase can have mostly resulted from the replacement of critical nutrients for bacterial proliferation.

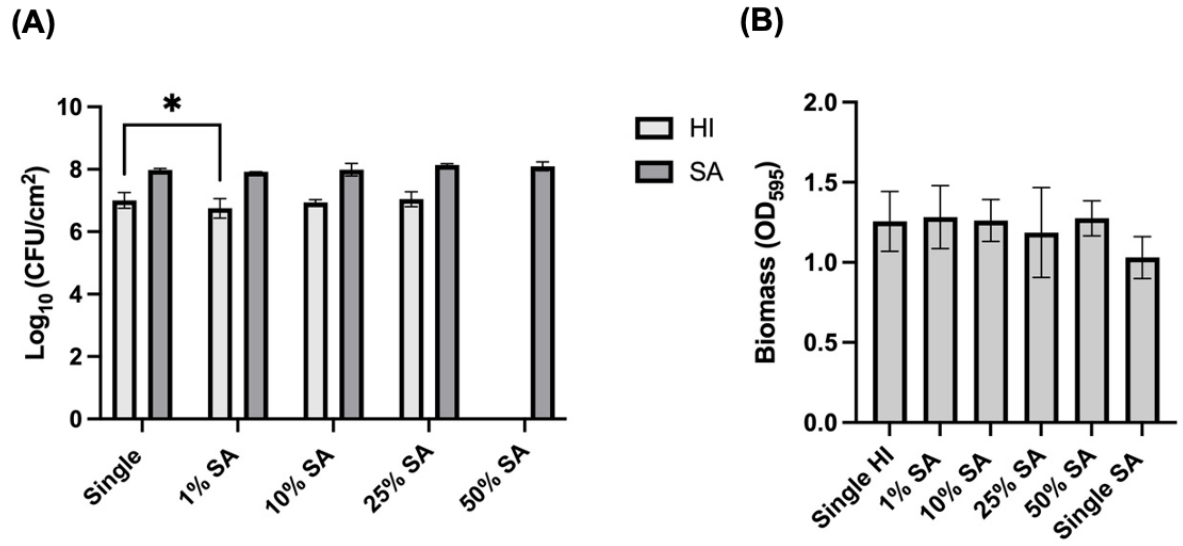

Figure 13 – Quantification of culturable *H. influenzae* and *S. aureus* (A) and total biofilm biomass (B) from 48 h double-species biofilms of *H. influenzae* with different initial concentrations of *S. aureus* (1%, 10%, 25%, and 50% V/V) with media replacement at 24 h. Statistically significant differences are represented by * $p < 0.05$. Standard deviations are indicated by error bars. Note: Due to unforeseen technical reasons, there is no data for the culturability of *H. influenzae* in *H. influenzae* biofilms with 50% of *S. aureus*.

Based on the results, it is mainly concluded that *H. influenzae* may have promoted the culturability of *S. aureus*, since in double-species of biofilms with a low percentage of *S. aureus*, there were practically the same amount of culturable cells in relation to the control biofilm. However, to assess this, a biofilm control with only *S. aureus* with an equal initial inoculum would have to be done. If *S. aureus* grows less, it can be confirmed that *H. influenzae* influenced the growth. If the growth of *S. aureus* remains equal, it

can be affirmed that *H. influenzae* had no influence on its growth, concluding that *S. aureus* simply grows fast, reaching the same cell density.

4.4 Influence of the Stepwise Colonization on Biofilm Formation

The process of biofilm formation is complex and involves multiple factors. One of the key factors that can impact biofilm formation is the order in which different microbial species attach to the surface. The presence of a first colonizing species can influence the adhesion and biofilm formation of a second species. When this happens, the first colonizing species may create a microenvironment less favorable for the growth and colonization of other species due to the production of inhibitory compounds, the consumption of necessary nutrients, or the physical blockage of the attachment sites of other bacteria. As a result, the second species may have difficulty adhering to the surface and forming a biofilm. However, the metabolic byproducts of the first colonizer can also support the growth of another colonizer, and the adhesion of one species can provide ligands that allow the attachment of others (Dunne, 2002; Margolis et al., 2010; Nair et al., 2014). In order to understand the influence of the presence of one colonizer species on the adhesion and biofilm formation of another, 48 h biofilms of one species were formed, where, after 24 h, the other species was introduced.

4.4.1 *S. aureus* as the First Colonizer

Results show that there were fewer culturable *H. influenzae* cells in the double-species biofilm where *H. influenzae* was the second colonizer for 24 h ($p < 0.01$) (Figure 14-A). However, these differences are not biologically significant because the difference between the number of culturable cells is minimal. In the quantification of biomass, although there was greater biomass production in the biofilm with two colonizers ($p < 0.05$) (Figure 14-B), given the associated error, this was also not considered biologically relevant.

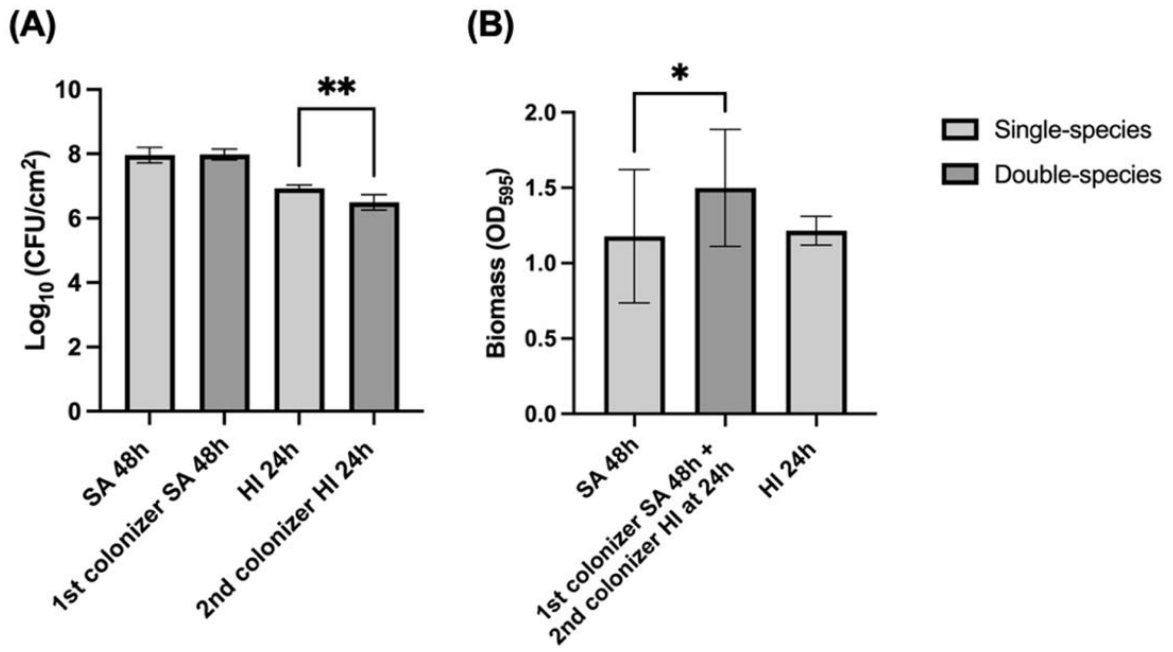

Figure 14 - Quantification of culturable *S. aureus* and *H. influenzae* cells (A) and quantification of biomass (B) from double-species biofilms in which *H. influenzae* was the second colonizer. Controls correspond to 48 h *S. aureus* and 24 h *H. influenzae* single-species biofilms. The statistical differences are represented by: * $p < 0.05$ and ** $p < 0.01$. Standard deviations are indicated by error bars.

The sequential colonization did not damage or enhanced the biofilm community since the number of culturable cells from the first and second colonizers is similar to the controls (Figure 14-A). Margolis and colleagues, in an *in vivo* study with newborn rats, obtained results that are relevant for this experiment. They verified that *S. aureus* and *H. influenzae* establish a synergistic interaction in the nasopharynx of rats and when grown *in vitro* in sBHI for 6 h. They report that, when *S. aureus* is the preceding colonizer, colonization by *H. influenzae* is more successful, reaching higher colony densities (Margolis et al., 2010). Given that, in our case, the culture lasted for 48 hours, over time some nutrients, such as hemin and NAD, could not be at the ideal concentrations to verify the growth of *H. influenzae*. In terms of biomass quantification (Figure 14-B), the slight increase achieved in biofilms with two colonizers compared to the 48 h single-species control biofilm may be related to the presence of additional cells and extracellular matrix due to the introduction of *H. influenzae* into the *S. aureus* biofilm.

4.4.2 *H. influenzae* as the First Colonizer

Subsequently, the reverse experiment was performed, where, after 24 h, *S. aureus* was introduced as a second colonizer in the pre-established *H. influenzae* biofilm. From the results obtained, it was verified that there was a smaller amount of culturable *H. influenzae* cells in the double-species biofilm ($p < 0.0001$) (Figure 15-A). However, these differences are not considered biologically significant because the difference between the number of culturable cells is minimal. The total biomass of the double-species biofilm was lower than that of the 48 h single-species control ($p < 0.05$) (Figure 15-B). On the other hand,

the double-species biofilm had a higher biomass production compared to the 24 h single-species control ($p < 0.0001$) (Figure 15-B).

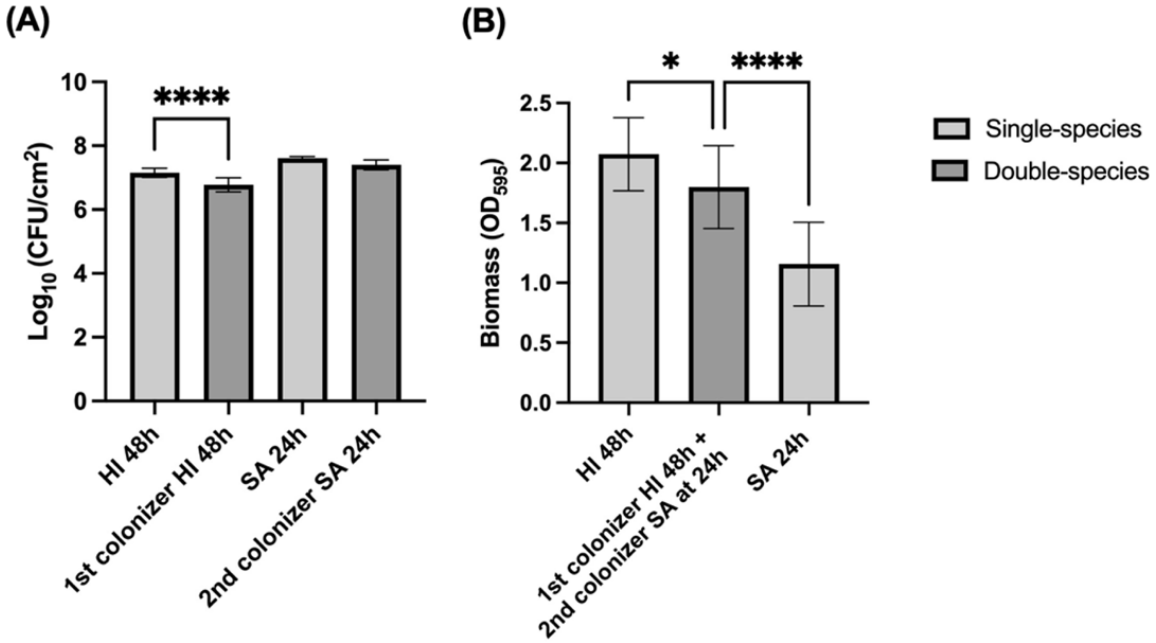

Figure 15 - Quantification of culturable *H. influenzae* and *S. aureus* cells (A) and quantification of biomass (B) from double-species biofilms in which *S. aureus* was the second colonizer for 24 h. Controls correspond to 48 h *H. influenzae* and 24 h *S. aureus* single-species biofilms. The statistical differences are depicted as: * $p < 0.05$ and **** $p < 0.0001$. Standard deviations are indicated by error bars.

The insertion of *S. aureus* into the pre-established *H. influenzae* biofilm after 24 hours had no influence on adhesion or biofilm formation because the number of culturable cells obtained was similar to the controls and the biomass was similar to the first colonizer. Given that *S. aureus* is known to be able to co-colonize with other bacteria, namely with *H. influenzae*, when it was introduced into the pre-established biofilm, it may have developed a cooperative relationship with *H. influenzae* (Nair et al., 2014). Although colonization of the secondary species had no significant effects on the existing biofilm of the other species, Esin and colleagues found that pre-colonization by *H. influenzae* promoted adhesion and biofilm formation of *S. aureus* in tympanostomy tubes (TTs), suggesting that there is a process active and synergistic in which *H. influenzae* promotes the attachment of *S. aureus*, such as cell-to-cell signaling, which is a critical early step in biofilm formation (Esin et al., 2015).

In conclusion, although the literature describes that the pre-establishment of the biofilm by one species can promote the adhesion, biofilm formation, and growth of other bacteria, in this study, it was seen that there was no influence of the colonizer species on the adhesion and biofilm formation of another species.

4.5 Interspecies Influence of Exoproducts on Biofilm Formation

Biofilm-forming bacteria can release EPS such as polysaccharides, small amounts of proteins, DNA, and other molecules into the surrounding environment that can help or harm other bacteria present in the biofilm, affecting their evolutionary dynamics (Nadell et al., 2008). In addition, through the QS cell-cell communication system, there is the production and release of AIs that provide a platform for intra- and interspecies crosstalk and the control of diverse bacterial activities, such as the formation and development of the biofilm, secretion of virulence factors, and survival in a constantly changing environment (Sahreen et al., 2022).

CFS is the liquid resulting from the removal of bacterial cells and contains metabolites from bacterial growth (some listed above) and residual nutrients from the medium used and has been studied mainly due to its antimicrobial potential against bacteria. Even though there is no literature about the effect of *S. aureus* and *H. influenzae* CFS on *H. influenzae* and *S. aureus* biofilms, respectively, some experiments with CFS against these strains have been done. For example, in a study by Coleman and colleagues, on the *in vitro* ability of alpha haemolytic streptococcus (AHS) and lactobacilli (LBs) from indigenous Australian children to inhibit the growth of respiratory pathogens, it was shown that LBs-free cells were effective in inhibiting *H. influenzae*, reducing the initial concentration by 3 logs (Coleman et al., 2022). Another study on the use of extracellular extracts of lactic acid bacteria (LAB) and bifidobacteria for the inhibition of *S. aureus* demonstrated that CFS, mainly produced by LAB, drastically inhibited the growth of the pathogen *S. aureus* (Hor & Liong, 2014). On the other hand, exoproducts can also benefit the biofilm. A study by Hou et al. found that the biofilm of an EPS-producing *S. aureus* ATCC 12600 exhibits a higher resistance to mechanical pressure than that of a non-EPS-producing *S. aureus* 5298, due to an immediate increase in polysaccharide content (Hou et al., 2018). Another example includes the reduction of penetration by some antibiotics into the *S. aureus* biofilm due to exoproducts (Yin et al., 2019). Additionally, the EPS matrix formed by *H. influenzae* provides a protective barrier that allows them to survive the stress caused by antimicrobial agents and the host's immune response (Tikhomirova & Kidd, 2013b).

In order to study the influence of exoproducts from one species on biofilms of another species, CFS from *H. influenzae* was added to 48 h *S. aureus* biofilms at 24 h and vice versa. The results show that the amount of culturable cells from HI biofilms with SA CFS and from SA biofilms with HI CFS was slightly higher ($p < 0.0001$) (Figure 16) when compared to the respective controls. However, these differences are not biologically significant, given the value proximity.

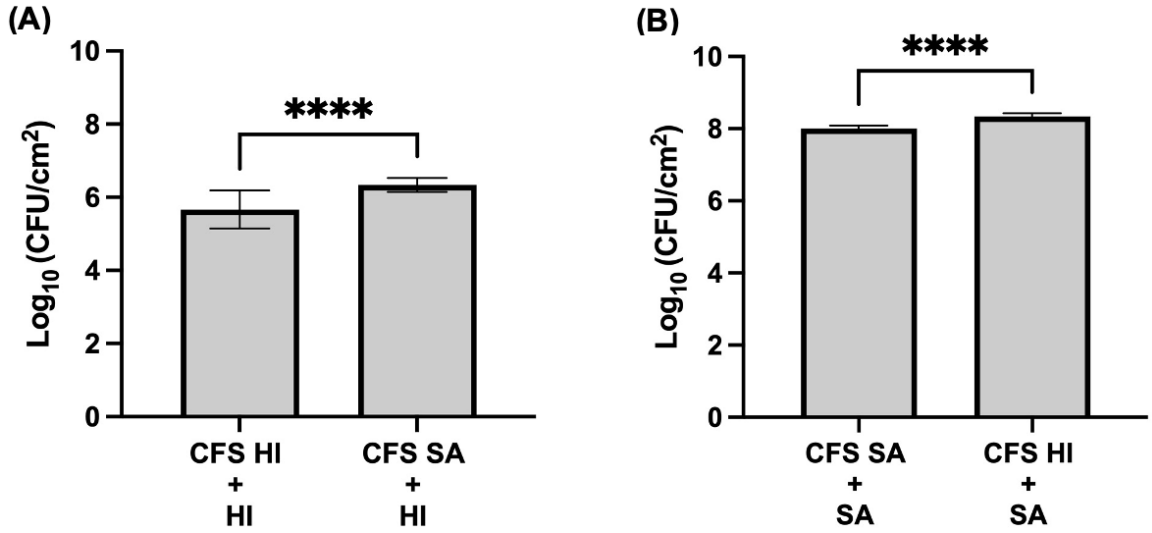

Figure 16 – Effect of SA CFS on HI culturable biofilm cells (A) and of HI CFS on SA culturable biofilm cells (B). The statistically significant differences are represented as: **** $p < 0.0001$. Standard deviations are indicated by error bars.

The bacterial viability of the 48 h biofilms may be related to the addition of CFS.This could be due to the reduction in the number of cells competing for nutrients, as a result of removing planktonic cells and adding CFS from 24 h biofilms that still have nutrients and EPS of the species (such as polysaccharides, proteins, eDNA, and lipids) that enhance the overall structure of the biofilm (Di Martino, 2018; Mani-López et al., 2022).

Compared to the controls, the exoproducts of *S. aureus* and *H. influenzae* did not have a negative or positive influence on *H. influenzae* and *S. aureus* biofilms, respectively (Figure 16). This may have occurred because exoproducts produced by biofilms during 24 h may not be at a high enough concentration to have an effect on the amount of culturable cells. Another factor is that *S. aureus* and *H. influenzae* biofilms may simply not be susceptible to the exoproducts produced by the other species.

4.6 Susceptibility *H. influenzae* and *S. aureus* to AMPs

The susceptibility of *H. influenzae* and *S. aureus* to four synthetic AMPs, namely temporin A, tachyplesin I, palm-KGK-PEG, and citropin 1.1, was assessed by the MIC and MBC (Table 4). Results show that the two species have different patterns of susceptibility to the chosen AMPs. Tachyplesin I showed the best bacteriostatic and bactericidal activity against both species. In *H. influenzae*, the second most active AMP was palm-KGK-PEG, followed by citropin 1.1 and, lastly, temporin A. In *S. aureus*, after tachyplesin I, temporin A was the second best, followed by palm-KGK-PEG, and finally citropin 1.1. To ensure that the assays were well performed, ciprofloxacin, which acts against both species, was used as a control.

Table 4 – Antimicrobial activity of temporin A, tachyplesin I, palm-KGKPEG, citropin 1.1, and ciprofloxacin against *H. influenzae* DSM 4690 (ATCC 33391, NCTC 8143) and *S. aureus* (ATCC 25923). MIC and MBC are expressed in mg/L.

	H. influenzae		*S. aureus*	
AMPs/Antibiotic	**MIC**	**MBC**	**MIC**	**MBC**
Temporin A	64	1024	16	128 - 256
Tachyplesin I	32	128	16 - 32	16 - 32
Palm-KGK-PEG	64	128 - 256	32 - 64	64 - 128
Citropin 1.1	64 - 256	256 - 512	64 - 128	128
Ciprofloxacin	< 0.015	0.06 - 0.125	0.001	> 0.002

According to EUCAST tabulated breakpoint values, the MIC values of ciprofloxacin indicate that *H. influenzae* and *S. aureus* are not resistant to this antibiotic (EUCAST, 2022b). This is in accordance to what is described for these strains , hence the assays were considered well executed (Chan et al., 2017; Kashef et al., 2020).

All AMPs under study have their mechanism of action targeting the bacterial membrane (Alves et al., 2016; Boland & Separovic, 2006; C. Liu et al., 2018; Mangoni & Shai, 2009). Tachyplesin I was the AMP that showed the greatest efficacy against *H. influenzae* and *S. aureus*, which might be explained by the fact that, in addition to targeting the bacterial membrane, it also acts at the intracellular level, targeting DNA (C. Liu et al., 2018; Yonezawa et al., 1992; Yu et al., 2020). For tachyplesin I, the MIC values were high (32 mg/L and 16 – 32 mg/L for *H. influenzae* and *S. aureus*, respectively) (Table 4) compared to MIC values found in the literature (0.8 - 12.5 mg/L) for Gram-negative (*H. influenzae*) and Gram-positive (*S. aureus*) bacteria (Xue et al., 2018). Repeated AMP freezing and defrosting cycles to make aliquots and its previous use can have made the AMP susceptible to degradation, contributing to lower antimicrobial activity and consequently higher MIC values (GenScript, 2022). On the other hand, it is possible that these strains may be resistant to tachyplesin I. However, this hypothesis would have to be confirmed through other assays.

Temporin A showed greater antimicrobial activity against *S. aureus* compared to *H. influenzae*. This result is consistent with the literature, since temporins are mainly effective and act quickly against Gram-positive bacteria. The activity against *S. aureus* presents MIC values (16 mg/L) within those mentioned in the literature, since generally the MIC of temporins ranges from 2.5 to 20 mg/L for Gram-

positive bacteria (Mangoni & Shai, 2009). For *H. influenzae*, the MIC value was substantially lower than what is published in the literature for Gram-negative bacteria (100-400 mg/L) (Rosenfeld et al., 2006).

Citropin 1.1 is considered to have a broad spectrum of action against both Gram-positive and Gram-negative bacteria (Boland & Separovic, 2006). In this study, citropin 1.1 demonstrated greater antibacterial activity against the Gram-positive bacteria *S. aureus*. In susceptibility researches with *S. aureus* (ATCC 25923), citropin 1.1 showed greater antimicrobial activity with lower MIC and MBC values (16 mg/L) (Jorge et al., 2017; Neubauer et al., 2017) than the values obtained here (MIC of 64 - 128 mg/L and MBC of 128 mg/L). The problems that may be associated with the higher MIC and MBC values obtained are the same as those previously reported for the AMP tachyplesin I.

Palm-KGK-PEG is a pegylated lipopeptide that demonstrated better antibacterial activity against *S. aureus* compared to *H. influenzae*. According to the literature, Palm-KGK-NH_2 (without PEG) has a potent antibacterial activity and is an excellent alternative to antibiotics since it has a low propensity for inducing microbial resistance (Alves et al., 2016; Avrahami & Shai, 2004). In addition, the association of AMP with PEG has the advantage of increasing water solubility, protecting the peptide constituents from protease degradation, producing larger conjugates that avoid rapid renal filtration to prolong circulation in the bloodstream, and further decreasing the potential effects of cytotoxicity against mammalian cells (Z. Cui et al., 2021). Similar activity of this lipopeptide without PEG against *S. aureus* was found in previous studies where, moreover, the activity against Gram-positive bacteria was also stronger compared to Gram-negative bacteria (Alves et al., 2016; Paduszynska et al., 2019).

Given that tachyplesin I demonstrated the best bactericidal and bacteriostatic activity against *H. influenzae* and *S. aureus*, it was considered the best AMP of the four AMPs tested.

Chapter 5. Conclusions and Future Work

COPD, a chronic, progressive, and incurable disease, is the third leading cause of mortality worldwide. The main causes of this disease are smoking, pollution and genetic deficiency of alpha-1 antitrypsin. Exacerbations, which are characterized by a worsening of COPD symptoms, occur recurrently and are mainly caused by bacterial infections. These infections are dangerous because they encompass the formation of polymicrobial biofilms, usually composed of NTHi with other bacteria, that often resist the administered antibiotics. The ineffectiveness of these drugs leads to a reduction in the quality of life of patients, comorbidities, and high mortality rates. As the presence of several species can aggravate bacterial resistance and infection resolution, knowledge on the bacterial interactions taking place is urgent to design better treatments.

This study examined some of the growth conditions of *H. influenzae* and *S. aureus* biofilms, both isolated and combined, including the effect of renewal the growth media and the influence of the different brands of growth media on the formation of biofilms. It also investigated the dynamics of bacterial interactions that occur within the double-species biofilms namely the impact of sequential colonization and of exoproducts, and the susceptibility of both species to AMPs.

The results showed that the lower number of culturable *H. influenzae* cells in 48 h biofilms without sBHI renewal at 24 h was probably due to nutrient depletion, given that when media is replaced at 24 h, the number of culturable cells is higher, but toxic metabolites produced by the bacteria itself or the existence of VBNC could also be involved. The results also revealed differences in the growth of *H. influenzae* when different media brands were used, which may be due to the quality of the medium, and that the repeated use of HI cryovials may impact bacterial quality and cause lower growth rates. It was also found that the growth of *H. influenzae*, both in single and in double-species biofilms, had less variability when it was grown in sBHI from Oxoid, making this the chosen brand for the following assays.

Regarding the dynamics of double-species biofilms with different initial concentrations of *S. aureus*, it was highlighted that *H. influenzae* and *S. aureus* appear to improve each other's culturability, but most differences encountered were small. Although it is reported in the literature that the density of *H. influenzae* increases when *S. aureus* is the previous colonizer, in this case, the colonizer species had no influence on the formation of the biofilm of the other species. Additionally, the results also showed that the exoproducts of one species had no influence on the formation of the biofilms of the other species, which could be due to the concentration of the exoproduct not being enough to trigger an effect, or the cells of the biofilms not be susceptible to the exoproducts produced by the other species. Finally, of the four AMPs tested, tachyplesin I showed the best bacteriostatic and bactericidal action against *H.*

influenzae and *S. aureus*, thus being more promising for further development of treatments against the double-species consortia.

Hopefully, the investigation of the growth conditions and of the interactions between these two species conducted in this work will provide the research team with valuable insights for the development of antimicrobial agents to treat infections caused by these bacteria.

To enhance the research conducted in this thesis, future work should include the discrimination of the microbial population of biofilms, namely viable, non-viable, and VBNC cells, for example through flow cytometry. Further investigations may be carried out, such as the identification of extracellular metabolites produced by biofilms when cultured with and without sBHI renewal, by mass spectrometry (MS). Another fundamental point would be the analysis of the spatial arrangement of the different species in the biofilm, where the fluorescence in situ hybridization (FISH) technique could be used.

For a more realistic study, biofilms could be grown in artificial mucus that mimics the mucus in the airways. It would be interesting to compare all the characterizations indicated above between the different growth media (sBHI and artificial mucus). Since, in chronic infections, most biofilms form small aggregates of cells not attached to a surface but embedded in host material, the biofilms, in addition to being formed on microtiter plates, could also be formed on alginate beads (Sønderholm et al., 2017). Alginate beads represent a simple and flexible model of *in vivo* biofilms. This would allow us to understand biofilm formation in different structures: microtiter plates (adhered biofilm) *vs* alginate granules (aggregated biofilm).

Finally, for the development of a possible drug, it would be essential to predict resistance for generations of *H. influenzae* and *S. aureus* to tachyplesin I. Resistance is tested for bacterial generations because a bacterial cell that is exposed to an antimicrobial agent can develop resistance and transmit this resistance to her descendants through her genetic material. By testing resistance in several generations of bacteria, it will be possible to determine how easily resistance is transmitted and how quickly it spreads within a population. In this way, the susceptibility test for bacterial generations could be performed. Given the significance of biofilms in this context, it would also be important to study the susceptibility of these bacteria to tachyplesin I when they are in single and double biofilms in order to gain a comprehensive understanding of how they are affected by this promising AMP.

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