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A scanning electron micrograph (SEM) showing a dense cluster of spherical Streptococci bacteria. The bacteria are arranged in a somewhat irregular, interconnected pattern, with individual cells appearing as small, rounded spheres. The background is a light gray, providing contrast for the darker, textured bacterial mass.

Antibiotic resistance and pathogenesis of Streptococci with focus on Group A Streptococci

FEIRUZ ALAMIRI

DEPARTMENT OF TRANSLATIONAL MEDICINE | LUND UNIVERSITY



Antibiotic resistance and pathogenesis of Streptococci with focus on Group A Streptococci

Feiruz Alamiri



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DOCTORAL DISSERTATION

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To be defended:

At 13:00 pm on December 18, 2020

In Agardhsalen at the Clinical Research Centre (CRC)

Jan Waldenströms gata 35, Malmö, Sweden

Faculty opponent

Professor Anna Norrby-Teglund

Karolinska Institute

Stockholm, Sweden

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Feiruz Alamiri



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Cover photo represents a microscopical image of non-encapsulated GAS bacteria formed on epithelial cells, captured by Maria Baumgarten (Lund University) and image modified by Zakaria Alamiri (Lund University)

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This thesis is dedicated to...

*My dear father who left this world on the 18th of April (2020),
eight months before my thesis defense.*

I love you dad...

Table of Contents

Preface.....	- 8 -
List of papers.....	- 9 -
Acknowledgements.....	- 10 -
Popular science summary (Swedish).....	- 14 -
Abstract.....	- 16 -
Introduction.....	- 17 -
Chapter 1: Human and bacterial organisms - friends or enemies?.....	- 19 -
Eukaryotes.....	- 19 -
Prokaryotes.....	- 20 -
Host - bacterial interactions.....	- 21 -
<i>Colonization and biofilm formation</i>	- 21 -
<i>Intracellular persistence</i>	- 22 -
<i>Opportunists</i>	- 23 -
Chapter 2: Antibiotic resistance and pathogenesis of streptococci.....	- 25 -
Streptococcal classification and pathogenesis.....	- 25 -
<i>Streptococcus pneumoniae</i> - “ <i>Captain of the men of death</i> ”.....	- 27 -
<i>Streptococcus pyogenes</i> – “ <i>The scarlet fever germ</i> ”.....	- 27 -
<i>Streptococcus agalactiae</i> - “ <i>The puerperal fever germ</i> ”.....	- 29 -
Antibiotic resistance mechanisms.....	- 29 -
<i>Altering penicillin binding proteins</i>	- 30 -
<i>Enzymatic methylation of ribosomes</i>	- 30 -
<i>Efflux pumps</i>	- 30 -
Antibiotic avoidance mechanisms.....	- 31 -
<i>Biofilm formation</i>	- 31 -
<i>Intracellular residence</i>	- 31 -
Chapter 3: HAMLET - a potential future therapeutic.....	- 33 -
HAMLET purification from human milk.....	- 33 -
<i>ALA purification</i>	- 34 -
<i>Conversion of ALA to HAMLET</i>	- 34 -
HAMLET activity in bacteria.....	- 35 -

<i>Methods used to study HAMLET activity in bacteria</i>	35 -
<i>HAMLET sensitivity</i>	36 -
<i>HAMLET potentiated antibiotic activity</i>	38 -
To conclude.....	40 -
Chapter 4: Biofilm formation and cellular uptake of <i>Streptococcus pyogenes</i>..	43 -
Surface colonization and biofilm formation in GAS.....	43 -
<i>Methods used to study GAS biofilms</i>	44 -
<i>Biofilm formation of GAS on epithelial cells</i>	47 -
Uptake and persistence of biofilm bacteria in epithelial cells.....	48 -
<i>Tools used to study intracellular persistence of GAS</i>	49 -
<i>Uptake and persistence of GAS within epithelial cells</i>	52 -
<i>Endocytic pathways involved in bacterial uptake</i>	52 -
<i>Epithelial cell responses to GAS infection</i>	53 -
To conclude.....	53 -
Chapter 5: Factors utilized during GAS pathogenesis of the respiratory epithelium	55 -
Strains and methodology used.....	56 -
Surface M protein.....	57 -
Hyaluronic acid capsule	58 -
The scarlet fever toxin SpeB	60 -
Streptolysin O and its co-toxin NADase	60 -
Streptolysin S	62 -
To conclude.....	62 -
Chapter 6: General summary and clinical significance of this thesis	65 -
Important highlights	65 -
Future plans.....	67 -
<i>In HAMLET</i>	67 -
<i>In GAS colonization and biofilm formation</i>	68 -
<i>In uptake and persistence of GAS bacteria</i>	68 -
Clinical significance.....	69 -
References	71 -

Preface

What a journey!

This PhD has been like a rollercoaster, with lots of ups and downs, but here I am today writing the final words in my thesis. I still remember during my master studies how my former teacher described the PhD process, by saying:

“The four years of PhD can be divided into three stages:

- 1. Getting familiar with the research area, planning the projects and developing the methods.*
- 2. Work starts flowing smoothly and all the results flood in.*
- 3. Putting everything into context in the form of manuscripts and finally ending this process with a fruitful thesis of great findings”.*

He was right, I could relate to all these stages during my PhD, but little did he mention how much stress and effort will be included in every stage and how many late nights and weekends will be spent working, reading or writing. I would define the PhD as a process where you put your personal life aside and prioritize your thesis simply because it’s what you’re passionate about. You end up living a PhD life that involves analysing and planning research related work at times you are supposed to take a break and relax, since you have a goal to reach i.e. the PhD degree.

While this describes a minor part of the PhD journey, the major part includes developing the critical thinking, presenting, writing and research skills. It’s fascinating how much I have developed as a researcher throughout these years and learned how to plan my own project and become an independent researcher. I realized the importance of collaborating with other skilful researchers to maintain great accomplishments and learn new techniques. Additionally, I discovered how passionate I am about teaching by supervising awesome students that I learned so much from. Finally, I spent great time with colleagues who became friends and collected unforgettable memories.

In these four years of research, I managed to design, plan and work on my projects. The outcome of these projects was four interesting papers that I describe in detail here and highlight the clinical importance of the obtained findings.

Finally, I hope these findings will be of importance in the clinical microbial field, especially in the process of treating respiratory infections caused by streptococci.

Sincerely, Feiruz Alamiri

6th of November 2020



List of papers

Paper 1

Alamiri F, Riesbeck K, Hakansson AP. 2019. *HAMLET, a protein complex from human milk, has bactericidal activity and enhances the activity of antibiotics against pathogenic streptococci*. Antimicrob Agents Chemother 63: e01193-19.

Paper 2

Alamiri F, Chao Y, Baumgarten M, Riesbeck K, Hakansson AP. 2020. *A role of epithelial cells and virulence factors in biofilm formation by Streptococcus pyogenes in vitro*. Infect Immun 88: e00133-20.

Paper 3

Alamiri F, Tang D, Malmstrom J, Hakansson AP. *Gene and protein expression profiles in biofilms of Streptococcus pyogenes expressing or lacking virulence factors*. In manuscript.

Paper 4

Alamiri F, André O, Nordenfelt P, Hakansson AP. *Internalization and persistence of Streptococcus pyogenes biofilm bacteria in epithelial cells in vitro*. In manuscript.

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Work on this thesis wouldn't have been possible without the person who actually provided this PhD position. My dear supervisor **Anders P Håkansson**, I am grateful to you for providing me the opportunity to become a member of your lab group. I need to admit that it was tough to email you every month for a full year asking you if you got the money for the position, however after all these years, I realized that professors do need a "small" reminder sometimes. During these 5 years in your lab, I learned so much from you and developed my research and writing skills. I also discovered my teaching skills when you gave me the opportunity to supervise students in lab. Thank you for always being there when I needed help and for all the guidance, support and inspiring ideas. I am grateful to you for providing me the freedom in research and sharing my ideas which helped me become the independent and skilful researcher I am today.

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Former and present lab members, **Caroline Bergenfelz**, my desk neighbour in the office, thank you for all the countless times you told me “bless you” every time I sneezed before the corona virus spread (didn’t dare to sneeze lately). Also, thank you for the fruitful discussions and guidance in the CBA assay. **Anki Mossberg**, thank you for guiding me when I started in lab, and also for providing HAMLET for paper 1. **Michelle Darrieux**, for your positivity, humour and the time you spent in lab. I tried to rescue the beautiful flowers you gave me but turned out I don’t have green hands.

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Master program in molecular microbiology at Lund University...

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My dear family, despite the tough times we went through, you always stood by my side and pushed me to do better and never give up. **Mom**, for your endless support, lovely heart, delicious food, and pride that I can clearly see in your eyes. **Zakaria** and **Khawla**, for all the late nights you drove me to/from lab, offering me dinner when I was lazy to cook after work, and for your children's (**Yahya** and **Adam**) endless love. **Reda**, for your sense of humour. **Aya**, for your creativity and eagerness to learn. **Kassem**, for your kindness.

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My dear family in law, thank you for your kindness, support, and care. **My father in law**, for your exceptional humour. **My mother in law**, for your sweet and loving heart.

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The Department of Translational Medicine, Faculty of Medicine, and Lund University for providing a comfortable environment, supportive and educational courses that helped me to develop and pursue my PhD studies.

The Royal Physiographic Society, the Swedish Research Council, and Alfred Österlund foundation, who funded this project and allowed me to conduct my research.

Finally, I would like to thank...

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~ Thank you all! ~

Popular science summary (Swedish)

Antibiotika-resistens utgör ett ökande hälsoproblem i världen och bakterier blir alltmer resistenta mot de antibiotika vi har att behandla med. Fler bakterier blir också resistenta mot mer än ett antibiotikum och behandling av infektioner med dessa typer av bakterier kompliceras av bristen på effektiva läkemedel vilket leder till ökade sjukvårdskostnader, längre vårdtider och en minskad chans att överleva resistenta infektioner. Ökad spridning av antibiotika-resistens bland streptokocker som *Streptococcus pneumoniae*, *Streptococcus pyogenes* och *Streptococcus agalactiae*, utgör en global fara för människors hälsa. Antibiotika-resistens i dessa bakteriearter sker via ett antal mekanismer bl.a. proteiner som bryter ner antibiotika, utflödespumpar som pumpar ut antibiotika utanför bakterien, strukturändring på bakteriemolekyler som minskar antibiotikas möjlighet att binda och m.m. Det krävs således nya behandlingsalternativ som bakterier inte bildar resistens mot, men då upptäcker och utveckling av nya konventionella läkemedel dröjer är molekyler från naturkällor ett annat alternativ. Ett exempel är bröstmjölk som är känd för sina antibakteriella egenskaper.

I denna avhandling presenterar vi ett protein-komplex som vi tidigare har upptäckt i bröstmjölk och som har förmågan att döda vissa bakteriearter. Komplexet heter HAMLET (Human Alpha-lactalbumin Made Lethal to Tumor cells) och består av proteinet alfa-laktalbumin som binder in fettsyran oleinsyra. Vi visar här att HAMLET-behandling av ovan nämnda streptokocker hämmar deras tillväxt och följaktigt leder till bakteriernas död. Förutom en direkt bakteriedödande effekt har HAMLET visat sig kunna öka aktiviteten av antibiotika mot bakterier som har utvecklat resistens, vilket leder till ökad känslighet mot antibiotikan och slutligen bakteriedöd. Vi visar här att streptokocker också är en bakteriegrupp vars antibiotikaresistens kan påverkas av HAMLET. Kombinationsbehandling av dessa bakteriearter med HAMLET och antibiotika som bakterierna är resistenta mot leder till ökad känslighet av bakterierna mot antibiotikan med resulterande bakteriedöd, oavsett resistensmekanism eller stam typ. Således utgör HAMLET en potentiell form av framtidsbehandling mot streptokockinfektioner.

Vissa bakteriearter skyddar sig från effekten av antibiotika genom att bilda biofilmer (organiserade bakteriesamhällen) när de koloniserar slemhinnor i kroppen eller tar sig in till människoceller (internalisering) och gömmer sig där under långa tidsperioder (persistens). Vi har tidigare sett att *Streptococcus pyogenes* (även känd som Grupp A Streptokocker, eller GAS) bildar biofilmer när de koloniserar keratinocyter in vitro och på ett okänt sätt tar biofilm-bakterierna in sig i cellerna utan att bli upptäckta eller dödade av cellen. I de flesta fall ses bärarskap utan symtom hos friska individer (5 - 20%), vilket eventuellt förklarar hur bakterierna sprider sig mellan individer. Men ibland orsakar bakterierna infektioner. GAS är en humanspecifik patogen som orsakar lokala (faryngit och ytliga hudinfektioner) såväl som systemiska (köttätande och toxinmedierade) infektioner. Återkommande

faryngit är ett hälsoproblem hos barn där GAS-bakterier gömmer sig inuti celler i halsmandlarna, och efter avslutad antibiotika-behandling, kan ta sig ut i svalget där de startar en ny infektion. I vissa fall slutar detta med en kirurgisk procedur där halsmandlarna opereras bort på grund av misslyckad behandling.

Baserat på denna kliniska information avser denna avhandling att förstå GAS-mekanismer under luftvägsinfektioner samt identifiera potentiella faktorer som GAS använder för att initiera dessa infektioner. Med hjälp av vår etablerade biofilmmodell och GAS-mutanter som saknar specifika virulens faktorer, undersökte vi biofilm-bildning i dessa bakterier, analyserade biofilmernas protein-reglering, och identifierade faktorer ansvariga för kolonisering och biofilmbildning. I en levande infektionsmodell undersökte vi mekanismerna bakom internalisering och persistens av GAS i luftvägsceller och med hjälp av mikroskopering, visualiserade och lokaliserade vi bakteriernas lokalisering inuti celler. Vi kom fram till att upptag av biofilm-bakterier i luftvägsceller är en gemensam mekanism bland GAS-bakterier och att biofilmbakterier stannar lika länge inuti celler, oberoende av stam typ. Med hjälp av mutanterna identifierade vi potentiella faktorer som krävs för internalisering- och persistens-processerna. Generellt är GAS faktorer som är inblandade i kolonisering, biofilm-bildning samt internalisering och persistens inom luftvägsceller möjliga behandlingsmål som kan användas för att utveckla nya strategier för att bota GAS-infektioner.

Erhållna resultat i denna avhandling har klinisk potential. HAMLETs antibakteriella effekter kan användas som en möjlig behandlingsterapi mot streptokockinfektioner. Vi har även undersökt GAS-mekanismer under luftvägsinfektioner samt identifierat inblandade faktorer som kan potentiellt användas som behandlingsmål mot dessa infektioner.

Abstract

Multi-drug resistant (MDR) infections remain the leading cause of death worldwide. MDR infections caused by *Streptococcus pneumoniae* (Spn), *Streptococcus pyogenes* (GAS) and *Streptococcus agalactiae* (GBS) are considered global threats to human health due to increased spread of antibiotic resistance and limited treatment options. In this thesis, we present the human milk derived HAMLET (Human Alpha-lactalbumin Made Lethal to Tumour cells) complex as a potential therapeutic alternative against streptococcal infections for its bactericidal and bacteriostatic activity against broth grown streptococci (Spn, GAS, or GBS). Adding to it, HAMLET potentiated antibiotic activity that renders antibiotic-resistant streptococci sensitive to the drugs they are resistant to, regardless of expressed serotype or antibiotic-resistance mechanism (target modification or efflux pumps).

Biofilm formation and intracellular residence are antimicrobial avoidance mechanisms that help GAS escape host- or antibiotic-killing mechanisms. After completed antibiotic treatment against pharyngitis, intracellular bacteria may re-emerge and cause recurrent infections, leading to treatment failure. This thesis aims to identify novel therapeutic targets during respiratory infections by investigating GAS mediated pathogenic mechanisms. As most biofilms were studied on non-representative abiotic surfaces, we used a well-established biofilm model mimicking the respiratory niche to show that biofilm formation on pre-fixed epithelial cells is common in GAS. Proteome analysis of biofilm bacteria helped us identify proteins involved during biofilm formation and show that highly down-regulated protein expression is needed to form highly functional biofilms. In a live cell infection model, we show that biofilm bacteria internalize and persist equally long among GAS strains within epithelial cells. Using these models along with GAS strains lacking or expressing known virulence factors, we identify the role of these factors during biofilm formation and uptake into respiratory epithelial cells by GAS.

Overall, the results obtained here are of clinical importance and could help in finding potential therapeutic strategies targeting streptococci during respiratory infections.

Introduction

“Folks are dying simply because there is no antibiotic available to treat their infections, infections that not too long ago were easily treatable” – Jean Patel at Centers for Disease Control and Prevention (CDC, 2017)

Multi-drug resistant infections (human diseases caused by bacteria resistant to more than one antibiotic) remain the leading cause of death worldwide resulting in ~ 700 000 deaths every year (2014). By 2050, the death numbers are expected to rise to 10 million if the antimicrobial resistance problem is not addressed [1]. The WHO (World Health Organization, 2014) outlined this problem as a serious and growing threat to global health that would lead the world into a post-antibiotic era of untreatable infections, if no solutions are provided [2]. It didn't take long until a CDC report (Centers for Disease Control and Prevention in USA, 2019) alarmed the arrival of the post-antibiotic era and hoped for a chance to combat the spread [3]. In the same report, multi-drug resistant streptococci (such as *Streptococcus pneumoniae*, *Streptococcus pyogenes*, or *Streptococcus agalactiae*) along with other bacterial types (species) were listed as threats for human health to which new treatment strategies are urgently needed [3].

Sadly, available treatment alternatives are limited, and discovering new antibiotics is time consuming. Therefore, finding new treatment alternatives from natural resources that bacteria can't become resistant to, might be a beneficial way to battle infections caused by these organisms. Understanding the bacterial lifestyle and identifying the mechanisms used by pathogens (a bacteria that cause damage to the host) to mediate infections could help in identifying new therapeutic targets to combat such pathogens.

The aim of this thesis will be outlined in six chapters covering the following aspects:

- **Chapter 1:** A brief introduction about living organisms and focus on possible interactions between these organisms.
- **Chapter 2:** Introduce streptococcal literature, highlight infections caused by these organisms and discuss mechanisms involved in disease development (pathogenesis), antibiotic resistance, as well as antibiotic- or host-avoidance mechanisms.

- **Chapter 3:** Present a compound obtained from human milk as a potential therapeutic alternative and determine its activity in antibiotic resistant streptococci.
- **Chapter 4:** Investigate mechanisms used by *Streptococcus pyogenes* to escape antibiotic- or host-mediated killing and determine their role during pathogenesis within the human respiratory tract.
- **Chapter 5:** Identify possible therapeutic targets involved in the pathogenesis of *Streptococcus pyogenes* within the human respiratory tract.
- **Chapter 6:** Sum up the thesis and highlight the clinical significance of the obtained findings.

Chapter 1: Human and bacterial organisms - friends or enemies?

“For the first half of geological time, our ancestors were bacteria. Most creatures still are bacteria, and each one of our trillions of cells is a colony of bacteria” – Richard Dawkins (1996)

Life on this planet arose more than 3.85 billion years ago in the form of living organisms that originated at different time points. Cells are the building blocks of living organisms and can be categorized into two groups, the eukaryotes and the prokaryotes [4-6]. The eukaryotic and prokaryotic cells are commonly formed of a shield protecting the cells interior (plasma membrane coating the cytoplasm) from external threats and contains protein producing factories (ribosomes) and genetic coding systems (DNA) [5-7]. Most organisms in both groups harbor a cell wall that covers the cell membrane and provides additional protection. Due to these common structures along with similarities in molecular organization and function, eukaryotes are thought to originate from prokaryotes [5]. However, differences in the size, shape, behaviour and composition of their corresponding cells do exist [4, 5, 7].

Eukaryotes

Eukaryotic cells are the building blocks of several eukaryotic organisms such as plants, fungi, animals (the human body for instance), and protozoa. Many eukaryotes are unicellular living organisms, made of one eukaryotic cell, however multicellular organisms originating from unicellular eukaryotes and containing more than one eukaryotic cell are present [4, 6, 8]. Depending on the organism, the size of these cells differs and range between 10-100 μm [5, 7]. Eukaryotic cells are composed of a membrane-bound nucleus containing DNA, a cytoskeleton forming the backbone of the cells, and a complex endomembrane system that consists of energy producing structures (organelles) [4, 7]. These cells are unique for their ability to carry out endocytosis, a highly energy requiring process to engulf particles from the surrounding environment [4].

Human eukaryotic cells have different shapes, functions and form different tissues [9]. The epithelial tissue, made of eukaryotic cells, is the shield that covers and

protects the interior of different organs from external threats and injuries. Epithelial cells form the lining of the skin (epidermis), and the respiratory, intestinal and urogenital tracts (mucosal epithelia) [10]. The epidermis of the human skin is mainly composed of epithelial cells termed keratinocytes (90%) that can also be found in the respiratory tract (oral mucosa), but to a less extent and with different gene expression profiles [11, 12]. The skin and mucosal membranes are mechanical barriers that together with other guarding cells (immune cells) form the first line of defense by preventing the invasion of foreign organisms into the human body [10].

Prokaryotes

In contrast to eukaryotes, prokaryotes are mostly made of single cell organisms that lack the eukaryotic cellular components (such as membrane bound nucleus and organelles) and are unable to perform endocytosis [4, 5, 7, 13]. The cell size of these microbes (organisms that can't be seen by a naked eye) is significantly smaller, ranging between 0.1-10 μm , and therefore special devices providing a magnified image of these organisms are needed, such as microscopes [5-7]. These organisms have the ability to conduct horizontal gene transfer, a process of taking up DNA from the surrounding environment and incorporating it into the prokaryotic genome [4]. Interestingly, eukaryotic uptake of prokaryotic DNA has been recently documented [14]. Archaea and bacteria are single cell prokaryotes that have similar cell shapes but differ in membrane components and gene expression mechanisms.

- **Archaea:** extreme organisms that are mainly found in harsh environments, but are also detected in the human body [6, 15, 16]. Archaea harbors unique genetic sequences and use a eukaryotic-like gene expression mechanism [17].
- **Bacteria:** organisms exhibiting different forms and functions depending on the bacterial species. Bacteria live in various environments (such as nature or hospitals) where they interact with other living organisms. Human (host) and bacterial interactions are common. Bacteria residing in different human body sites behave differently and, in some cases, become pathogenic and cause damage in the host thereby initiating infections [16, 18]. Depending on their peptidoglycan content (building block of bacterial cell wall), most bacterial species can be divided into two sub-groups termed gram negative (thin peptidoglycan layer) or gram positive (thick peptidoglycan layer) [19].

Host - bacterial interactions

Classification of bacteria based on their ability to cause disease is not optimal and is rather complex. Within the same host, certain bacterial species behave differently depending on the adapting body site (niche) and the body's defense system (immune system). Sometimes human death can be caused by a massive immune response to the presence of a toxic bacterial substance (toxin), rather than the bacteria itself. Therefore, the proper terminology would be the one based on what kind of damage an interaction between bacteria and human can cause. In research, different terms are given to bacteria depending on their lifestyle, behaviour and function within the human body, however these terms do not always apply to all bacterial species [18, 20]. To better understand the concept of this thesis, we have used some of these terms (colonizer, commensal, opportunist, pathogen) to describe the lifestyle and function of the studied bacteria, but it should be kept in mind that this terminology does not always apply in the microbial world.

Colonization and biofilm formation

Colonization is the state where a foreign microbe enters the human body through different paths (transmission routes) and stays for a variable period of time in certain niches (such as skin, mouth, nose, or intestinal tract) [18]. Common bacterial transmission routes include (1) the fecal-oral route where food or water contaminated with fecal material of an infected person is ingested by another person, or (2) the close or direct contact with respiratory aerosols (droplets containing bacteria) that spread through sneezing or coughing, and originate from a person colonized with the microbe [21, 22].

Colonizers are bacteria residing silently with minimal effect on cell surfaces of the human body (asymptomatic colonization) and once adapting with other microbes in the colonizing niche become commensals that in some cases benefit the human body (member of the normal flora) [18]. Within the host, actively growing colonizers become pathogens by triggering microbe-mediated immune responses that damage the host over time and initiate infections. Generally, infections start due to dual responses by the host and the colonizing pathogen where virulence factors (bacterial tools allowing bacteria to replicate and spread within a host by avoiding host defenses) exposed or released by the bacteria are sensed by surface proteins (receptors) on human cells. These cells in turn release a set of signalling (inflammatory mediators) or toxic proteins (anti-microbial peptides) to kill the pathogen. The final outcome of this response is either microbial eradication or cellular invasion by the colonizer that survives for longer periods of time (persist) within the host [18, 23, 24].

Around 65% of bacterial infections are associated with biofilm formation [25]. Biofilms are organized complex three-dimensional structures that form on epithelial

cell surfaces upon bacterial colonization. Several steps are involved during biofilm formation, briefly, (1) bacteria attach to cells via surface exposed bacterial and cellular receptors, (2) accumulate and start multiplying and dividing to form small groups of bacteria (aggregation and microcolony formation), (3) communicate and signal other bacteria within the same microcolony to reach the required microbial density and produce gel-like structures termed extracellular matrix (ECM) so that a mature biofilm structure can be maintained, and (4) dissociate from biofilm structures (dispersal), disseminate and colonize other sites within the human body [25, 26]. Biofilm formation have been detected in both gram-negative and gram-positive bacterial species as a lifestyle to maintain extended survival within the host by avoiding host- or antibiotic-mediated killing [27-30].

Intracellular persistence

Most human pathogens colonize the external epithelial cell surface, whereas some manage to invade epithelial cells by manipulating cellular uptake proteins involved in endocytosis to their advantage, thereby entering cells (internalization) and persisting intracellularly without being detected or killed by the host [31]. Depending on the uptake mechanism used, endocytosed bacteria could end up in different intracellular structures such as endosomes, lysosomes, invasomes or other vacuoles. Bacterial elimination in these compartments is mediated by the acidic environment and toxic molecules present within these structures. However, some bacterial species adapt or modify the vacuolar environment and stay there, whereas others manage to escape these vacuoles and persist within the cytosol for longer duration of time [31-36].

Bacterial uptake mechanisms can be divided into two groups, (1) general uptake mechanisms using proteins commonly involved in all uptake mechanisms such as dynamin (vesicle formation), actin or microtubulin (cytoskeleton proteins), and (2) specific uptake mechanisms using certain proteins or structures that is not commonly used among bacteria during uptake such as the protein clathrin (coated vesicle formation), β 1-integrin (adherence to cells) or talin-1 (links integrin to cytoskeleton), or lipid-raft structures (cholesterol dense regions in plasma membrane that mediate signal transduction), respectively [31, 37-39].

Depending on the bacterial species and their corresponding pathogenesis mechanisms, cellular entry and intracellular lifestyle varies. Uptake of bacterial aggregates (group of bacteria) in *Bartonella henselae* (*B. henselae*) into invasomes of endothelial cells via actin, talin-1 and β 1-integrins have been documented [31, 34, 35]. On the other hand, uptake of individual bacteria has been shown to use different uptake mechanisms among which the trigger mechanism or the zipper mechanism are few examples. Trigger mechanism is a macro-pinocytosis process where bacteria via several signalling events (signalling cascade) manage to rearrange actin and remodel the host to form structures (membrane ruffles) that

facilitate bacterial uptake [31]. The bacterial complex termed type III secretion system (T3SS) in the gram-negative species *Shigella flexneri* (*S. flexneri*) or *Salmonella typhimurium* (*S. typhimurium*) triggers this mechanism. The T3SS directly injects virulence factors across the bacterial-host membranes into the host's cytoplasm that further help bacteria modulate the cellular cytoskeleton (via actin) thereby facilitating bacterial adherence, invasion and colonization of these cells [31, 40].

The zipper uptake mechanism is a receptor mediated process requiring clathrin and actin mainly. This mechanism is initiated by direct contact between bacterial ligands (virulence factors) and cell receptors that may or may not require lipid rafts (depending on the expressed bacterial ligand and cell receptors), consequently initiating cell membrane zippering around the bacteria that activate a set of signalling cascades, finally leading to bacterial uptake [31]. The gram-positive bacterium *Listeria monocytogenes* (*L. monocytogenes*) manages to enter cells via the zipper mechanism. Once inside cells, it escapes the membrane-bound vacuoles into the cytoplasm by expressing a certain type of cholesterol dependent cytolysin (CDC) termed listeriolysin O (LLO), and use actin to move within the cell thereby mediating prolonged intracellular persistence and protection against host-mediated killing [31, 36, 41].

Opportunists

Commensals co-exist peacefully with other microbes within the same niche without causing any harm or benefit. Commensals that provide nutrients, vitamins, protection against invading pathogens, and degrade food that the body is incapable to digest, are part of the normal flora. The normal flora, mainly harboring gram-positive species, form around 0.2 kg of the human body weight that consists of equal numbers of human and bacterial cells [16, 18, 42].

Under certain circumstances (such as environmental signals or weak immune systems) or when present outside their niche, commensals can become pathogenic (opportunists) and cause opportunistic infections that in some cases become severe and lead to human death [16, 43, 44]. For instance, intact human skin is the physiological niche for the gram-positive commensal *Staphylococcus epidermidis*, where it resides without causing any harm. However, when contaminating skin wounds, the commensal starts expressing a set of virulence factors and becomes pathogenic thereby causing cell damage and initiating skin infections that in some cases lead to sepsis if the pathogen reaches the blood stream (systemic infection of vital organs needed for human survival) [44, 45]. Similarly, *Streptococcus pneumoniae* and *Streptococcus agalactiae*, are other gram-positive commensals that have the ability to induce respiratory-, skin-, or brain-infections, or sepsis when leaving their physiological niche (nasopharynx or intestinal tract, respectively) [28, 30, 44].

Chapter 2: Antibiotic resistance and pathogenesis of streptococci

“To let a sad thought or a bad one, get into your mind is as dangerous as letting a scarlet fever germ get into your body. If you let it stay there after it has got in, you may never get over it as long as you live” ~ Frances Hodgson Burnett (1911)

During the 17th - 18th century, Europe and North America suffered from a scarlet fever epidemic causing high mortality numbers and implementing quarantine on patients exhibiting common symptoms such as fever, sore throat and rash. During the epidemic, small organisms were discovered by Theodor Billroth (in 1874), who named them Kettenkokken (or streptococci) and described them as berries present alone (coccus in Greek), in pairs (diplococci), or in the form of twisted chains (streptos in Greek) of four or more than twenty links. However, it was not until the 20th century when streptococci were identified as the etiological agent of this disease [46]. Moreover, during the 18th – 19th century, another outbreak of puerperal fever (postpartum infection) hit the same geographic regions mostly affecting women and newborns during childbirth. It was later shown that the affected women and newborns acquired the disease from healthcare workers who had been in contact with infected patients, and since then the importance of hand washing and sanitizing among healthcare workers have been noticed [10, 46]. The etiological agent of the epidemic was not identified until 5 years later after its discovery, by Louis Pasteur who drew a diagram of the dangerous chain-forming streptococci and pointed them out as being the main cause [46].

Streptococcal classification and pathogenesis

Since their discovery, several attempts have been made to classify streptococci into sub-groups. The first classification divided these bacteria into three groups based on their ability to lyse red blood cells (hemolysis) and form a discoloration zone around bacterial colonies grown on blood agar plates. These groups were termed alpha hemolytic (green zone of hemolysis), beta hemolytic (clear zone of hemolysis), or gamma hemolytic (no hemolysis) (**Fig. 1**). Later a new classification system was designed by Lancefield (in 1933) that was based on differences in surface antigens

(proteins inducing human immune responses), thereby splitting streptococci into groups designated with letters from A to X. For instance, group A involves bacteria from human diseases, group B contains bacteria isolated from bovine and dairy sources, and so on. Lancefield used a serological technique to identify and group bacteria, by which group-specific antigens were first extracted from streptococci through hot-acid extraction and then precipitated with serum containing group-specific antibodies (anti-sera). However, this classification did not apply to all streptococcal species since the group antigen of certain streptococci such as *Streptococcus pneumoniae* or viridans streptococci failed to bind to these antisera and therefore no grouping was assigned to these species [46, 47]. Recently, a more specific classification based on genomic homologies of the 16S ribosomal RNA (identified through sequencing) was designed and 55 streptococcal species were identified and grouped together [48]. Species within these groups were further classified into sub-groups (serotypes) based on structural similarities of antigens (other than the group antigens) expressed on the bacterial surface, such as the polysaccharide capsule antigen (shield covering the bacterium) in *Streptococcus pneumoniae* and *Streptococcus agalactiae*, or the surface M protein in *Streptococcus pyogenes* [46, 49, 50]. Asymptomatic colonization of certain parts of the human body by these species is common and their attack rate is usually low [28, 30, 51]. However, as described below, infections caused by these species have been detected that in some cases lead to human death.

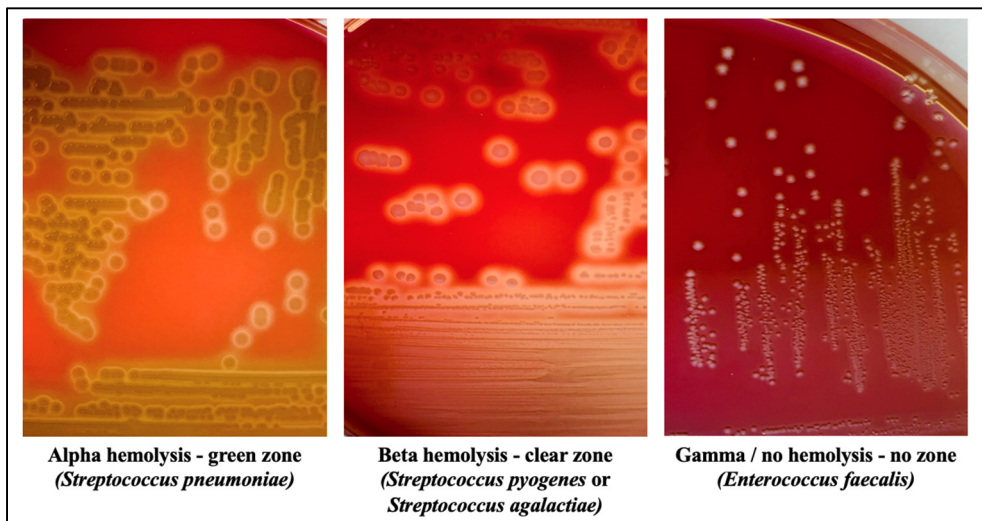


Figure 1. Streptococcal hemolysis on blood agar plate.

***Streptococcus pneumoniae* - “Captain of the men of death”**

“The captain of the men of death” (William Osler, 1918) or the “pneumococci”, are terms given to the human commensal *Streptococcus pneumoniae* (Spn) that commonly colonizes the nasopharyngeal mucosa in adults (5-20%) and children (20-50 %) [28, 44]. To date, 100 serotypes of Spn have been identified and classified based on their virulence-related polysaccharide capsule [49]. Available vaccines target this capsule and provide protection against infections caused by certain pneumococcal serotypes. However, this isn’t enough to protect against pneumococcal infections since serotypes not covered by the vaccines are still able to cause diseases. Also, certain isolates (strains isolated from infected patients) have the ability to incorporate capsule encoding genes from other pneumococcal strains into their genomes via horizontal gene transfer and further express a new capsule type (common in pneumococci), or switch into different serotypes by altering the genetic sequence of the *wciP* gene by point mutations [52-55].

Individual pneumococci grow in broth (planktonic bacteria) in the shape of diplococci or short chains [44, 53]. During nasopharyngeal colonization, pneumococci adhere to epithelial cells and form complex biofilm communities coated with ECM. The pneumococcal ECM contains polysaccharides, proteins and DNA, and forms a protecting shield against external threats (such as antibiotics, antimicrobial peptides, immune cells) [28, 53]. Upon certain stimuli (virus infection for example), pneumococci dispersed from biofilms travel to niches within the human body that they don’t normally colonize (non-physiological niches), thereby mediating opportunistic infections [28, 53]. Pneumococcal infections range from being mild middle ear infections in children (otitis media) to lethal infections such as lung- (pneumonia), blood- (sepsis), and brain-infections (meningitis).

In 2016, lower respiratory tract infections killed around 650 000 children (< 5 years old) and 1.1 million adults (older than 70 years), globally. In these infections, pneumococci was the leading cause of lower respiratory tract morbidity and mortality as compared to other disease causing agents [56, 57]. The CDC in USA considers this multi-drug resistant pathogen as a serious threat due to the alarming mortality rates every year and rapid spread of drug resistance to clinically relevant antibiotics targeting the bacterial cell wall (β -lactams, such as penicillin), or protein synthesis (macrolides such as erythromycin or lincosamides such as clindamycin) among Spn [3]. Globally, the WHO listed the penicillin-non-susceptible pneumococci in the pathogen priority list for which new treatment alternatives are urgently needed [58].

***Streptococcus pyogenes* – “The scarlet fever germ”**

Hence its name, the pus forming (pyogenes in Greek) microbe *Streptococcus pyogenes* (also known as group A streptococci, or GAS) is a versatile human

pathogen that causes around 18 million severe infections annually (in children and adults) of which 517 000 cases are fatal [46, 59, 60]. Colonization of mucosal linings by GAS is highly linked to microcolony formation that further progress into complex three-dimensional biofilms [29, 51, 61-63]. Dispersal of GAS biofilms is also documented, where dispersed bacteria spread to other body sites and cause mild to severe infections [64].

Infections caused by GAS depend on its colonization niche within the human body and involves multiple organ systems. Colonization of the oropharynx (mouth) can result in pharyngitis (or “strep throat”), the most common type of GAS infection resulting in up to 600 million infections worldwide, annually. Scarlet fever is commonly associated with GAS mediated pharyngitis [46, 59, 60, 65, 66]. However, asymptomatic colonization of the oropharynx have been detected in 20% of children [51]. When colonizing the respiratory tract, GAS mediates respiratory infections in the lungs (pneumonia) or ears (otitis media) [67-69]. On the other hand, colonization of injured skin surfaces can lead to impetigo and other deeper skin infections, such as necrotizing fasciitis (also known as the flesh-eating disease) where biofilm structures have been detected [60, 70].

To date, more than 200 GAS serotypes have been identified and classified by the surface M protein (a major virulence factor). An individual is often colonized or infected with more than one serotype during their lifetime. However, recurrent pharyngitis (repeated pharyngeal infections) with the same serotype has been documented in children [46, 63, 71-73]. Tonsil specimen taken from patients with recurrent pharyngeal infections revealed the presence of intracellular GAS bacteria, which could explain the reason behind treatment failures of these infections [74]. Unfortunately, preventing GAS infections is not possible since no vaccine has been developed yet. The reason behind that is the presence of several stakeholders such as high structural variability of surface antigens or lack of relevant animal models (since it’s a strict human pathogen) [75].

During infection, the treatment of choice is penicillin that kills GAS bacteria residing outside cells. However, an alternative treatment is needed for patients suffering from penicillin allergy, penicillin resistant infections (rare, detected only in two isolates), or penicillin non-reachable infections caused by intracellular GAS isolates [72, 76]. Antibiotics targeting the protein machinery (erythromycin or clindamycin) by entering the intracellular milieu, are next chosen to combat GAS infections [77-79]. Unfortunately, resistance to erythromycin and clindamycin is rapidly spreading among GAS isolates and the pathogen is considered a concerning threat to which new treatment alternatives are needed [3]. Upon repeated treatment failures of recurrent pharyngeal infections, a surgical procedure to remove the infected tonsils (tonsillectomy) is considered [71].

***Streptococcus agalactiae* - “The puerperal fever germ”**

The causative agent of puerperal fever, *Streptococcus agalactiae* (also known as group B streptococci, or GBS), is part of the vaginal microflora colonizing around 30% women worldwide [30, 44, 80]. The natural reservoir for this commensal is the human gastrointestinal tract, that possibly is the source of vaginal colonization. Similar to *Spn*, capsular serotyping has identified and classified 10 GBS serotypes (Ia, Ib, II – IX) and biofilm formation has been documented in these species [30, 50].

Up to 50% of pregnant women colonized with GBS, transfer the pathogen to their neonates during pregnancy or delivery, which consequently lead to neonatal infections. As indicated by a study in 2015, the final outcomes of most pre-neonatal infections are still births (57 000 neonates) or pre-term births (3.5 million neonates), and severe neonatal infections that in most cases are lethal (90 000 infant deaths) [81, 82]. GBS infections developed in newborns during the first week of birth (early-onset disease, EOD) include pneumonia and sepsis (up to 6%), whereas those developed in later stages (late-onset disease, LOD) include severe meningitis. GBS is therefore classified as the leading cause of neonatal infections worldwide for which vaccine development is urgently needed [30, 50, 82, 83]. Several vaccine candidates targeting GBS are present today but are still in the preclinical and clinical trial phase [84].

Opportunistic GBS infections in adults are common, and include sepsis, brain-(meningitis), bone- (osteomyelitis), or heart-infections (endocarditis), as well as other non-invasive diseases. Patients at high risk are those suffering from diabetes, malignancies (cancer), or a weak- (elderly) or impaired-immune system (immunocompromised) [30, 50].

To prevent the risk of EOD, intrapartum antibiotic prophylaxis (IAP) is used to reduce vaginal colonization by GBS in pregnant women that during labor are given intravenous antibiotic treatment with penicillin or clindamycin (in case of penicillin allergy). However, a limitation to the IAP preventive method is that it does not prevent LOD, stillbirth or prematurity caused by GBS [84, 85]. Penicillin resistance has emerged, and erythromycin and clindamycin resistance is rapidly spreading among GBS isolates, which further classify this pathogen as a concerning threat [3, 82, 86, 87].

Antibiotic resistance mechanisms

Generally, antibiotic resistance in bacteria is mediated by one or multiple mechanisms. Among these mechanisms are those that cleave the antibiotics and render them inactive (enzymes), alter the antibiotic target (by enzymatic activity or gene modification; mutation), or pump the antibiotic out of the bacterial cell (efflux

pumps) [88, 89]. Using horizontal gene transfer, bacteria acquire resistance by uptake of DNA fragments containing genes encoding resistance mechanisms (mutations, enzymes or efflux pumps) from their surroundings and incorporate them into their genome. This is common in bacteria present in biofilms and living in close proximity or having a direct cell-to-cell contact with other bacterial species colonizing the same niche within the human body [27, 90]. In streptococci (such as Spn, GAS or GBS), resistance to antibiotics (β -lactams, macrolides, or lincosamides) is mediated by the following mechanisms:

Altering penicillin binding proteins

The main targets of β -lactams (such as penicillin) are proteins involved in peptidoglycan synthesis (cell wall), known as penicillin binding proteins (PBPs). The three main PBPs involved in β -lactam resistance are the PBP1a, PBP2b and PBP2x. Altered gene sequences (by mutations) and mosaic structure of PBPs are the main mechanisms conferring reduced penicillin sensitivity by blocking binding of the antibiotic to the streptococcal cell wall [76, 82, 91, 92].

Enzymatic methylation of ribosomes

Macrolides (erythromycin), Lincosamides (clindamycin) and Streptogramin B belong to the MLS group of antibiotics that use the same mode of action in bacteria. MLS antibiotics bind to same targets in bacterial ribosomes and thus inhibit protein production. Cross-resistance to these antibiotics is mediated by bacterial expression of Erm (erythromycin ribosome methylation) enzymes encoded by the *erm* gene and that add methyl groups to the 23S ribosomal RNA [82, 93]. To date, a number of Erm gene variants have been identified in streptococci [94]. These include:

- ErmA (also known as ErmTR) is an enzyme whose expression is induced by MLS antibiotics but can also be constitutively expressed. In GAS, ErmTR is widely distributed and its resistance levels depends on the simultaneous presence of drug efflux pumps [93, 95, 96]. This enzyme is also present in MLS resistant isolates of GBS and pneumococci [97, 98].
- ErmB is the widely spread, pre-dominant Erm enzyme, that is present in most streptococci (such as Spn, GAS, or GBS) and is associated with high resistance levels to MLS antibiotics. Similar to ErmA, the expression of ErmB in streptococci is either constitutive or MLS inducible [93, 95-97, 99].

Efflux pumps

Another form of acquired macrolide resistance is the presence of efflux pumps that expel the antibiotic outside bacteria. Two types of efflux pumps known as MefA

and MefE have been identified. These pumps are encoded by genetic elements sharing DNA (90 %) and amino acid (91 %) sequence homologies and are therefore considered a single class pump, termed MefA. The expression of MefA is accompanied by the expression of an energy dependent transporter termed MsrD that is thought to function with MefA as dual efflux pumps [93, 94]. MefA was first documented in GAS and Spn, later erythromycin inducible co-expression of MefA and MsrD was discovered in pneumococcal isolates and was also detected in GAS isolates [66, 96, 100-102]. On the other hand, macrolide resistant GBS isolates have only been found carrying the MefA efflux pump with no traces of MsrD [103, 104].

Antibiotic avoidance mechanisms

Reduced antibiotic sensitivity can be mediated by thick cell walls in bacteria that block antibiotic penetration [88, 89]. Moreover, certain bacterial lifestyles can also mediate reduced antibiotic sensitivity which help bacteria survive antibiotic-mediated killing. Note that these mechanisms also confer a form of antibiotic resistance, but to distinguish them from the direct resistance mechanisms (mentioned above), we will call them “antibiotic avoidance mechanism” hereafter. Below are examples of antibiotic avoidance mechanisms streptococci use to maintain their survival during infection.

Biofilm formation

No or slow bacterial growth protects bacteria (dormant persister cell) from the killing effect of antibiotics targeting actively growing bacteria. This mechanism is known as tolerance by which dormant persister cells shut down (down-regulate the expression) antibiotic targets [25, 105, 106]. Slow growing persister cells are present in biofilms, and along with the impermeable biofilm structure, provide protection against antibiotics [25, 63, 88, 106]. During colonization of human epithelial surfaces, streptococcal species (Spn, GAS and GBS) tend to form biofilms as a protection and survival mechanism [28-30].

Intracellular residence

Beside the protection from host-mediated killing, intracellular residence also protects the pathogen from antibiotics where intracellular pathogens, after finished antibiotic treatment, manage to get their way out of cells (re-emerge) and cause recurrent infections. One example is the recurrent pharyngeal infections caused by the human pathogen GAS. In these species, uptake and persistence of biofilm bacteria have been observed (described in detail in **chapter 4 – 5**), presumably correlating to treatment failures of these infections [18, 29, 72].

Chapter 3: HAMLET - a potential future therapeutic

“One sometimes finds what one is not looking for” – Sir Alexander Fleming (1945)

In 1995, a protein-lipid complex was discovered by serendipity when anti-adhesive properties of human milk were studied in respiratory epithelial cells infected with pneumococci. Leaving healthy epithelial cells intact, this human milk complex has the potential to kill tumor cells (tumoricidal activity) [107]. It is composed of the highly abundant human milk protein alpha-lactalbumin (ALA) as well as the unsaturated fatty acid oleic acid. Despite ALA being a whey protein, the human milk complex containing this protein is detected in the casein fraction. Mixing ALA and oleic acid in a specific way in the laboratory provides a similar complex as the one isolated from the casein fraction in human milk, and the complex is therefore named as the Human Alfa-lactalbumin Made Lethal to Tumor cells (HAMLET) [108].

HAMLET purification from human milk

The HAMLET complex, as a whole, is not present in human milk. However, ALA and oleic acid that are naturally present in human milk are components forming this complex. Purification and partially unfolding ALA by the removal of its calcium ion (Ca^{2+}) and binding the unfolded protein to oleic acid, are major steps involved in HAMLET production (**Fig. 2**) [108, 109].

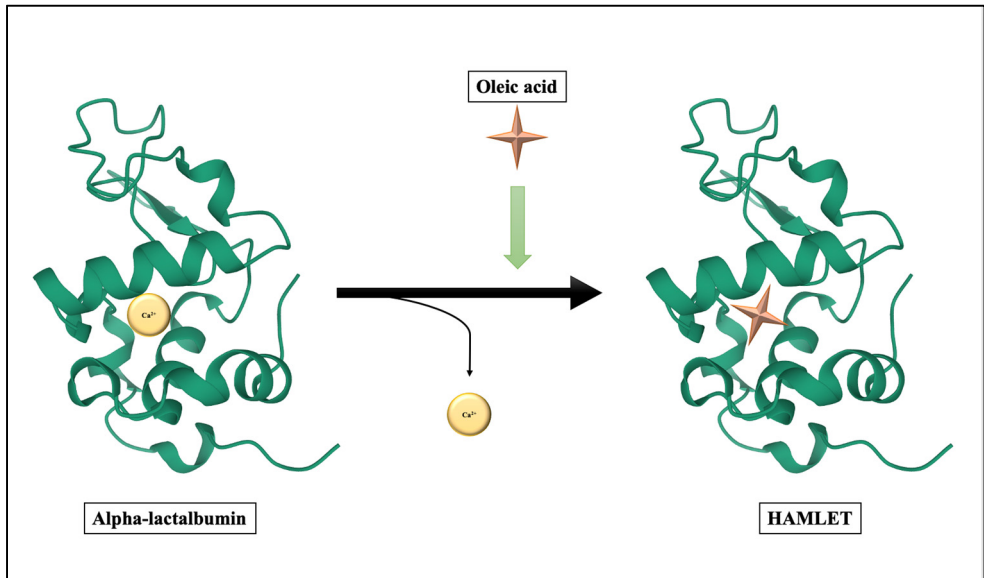


Figure 2. Conversion of human alpha-lactalbumin (PDB ID = 1A4V) to HAMLET [110, 111].

ALA purification

To purify ALA from human milk, the milk is first centrifuged at high speed to remove fat globules. In the defatted milk, proteins other than ALA are then precipitated with salt and the ALA is concentrated and made hydrophobic (dislike water) by massive exposure to a calcium removing molecule termed EDTA (ethylene diamine tetraacetic acid) that remove the strongly bound calcium, rendering the protein partially unfolded (Apo-ALA). Exposing hydrophobic domains enable the protein to bind tightly to a hydrophobic matrix on the separation column during chromatography (separation technique). When changing to Ca^{2+} containing buffer, ALA will revert back to its native hydrophilic (prefers water) and folded conformation, detach from the matrix, and elute from the column.

Conversion of ALA to HAMLET

As HAMLET is made of partially unfolded ALA stabilized with milk-specific fatty acids, conversion of ALA to HAMLET involves first converting the purified native ALA into apo-ALA by EDTA treatment along with oleic acid (18-chain unsaturated fatty acid) binding to the ion exchange matrix used for conversion. Then apo-ALA is added to the oleic acid containing matrix, so that both components bind to each other and convert into HAMLET. After washing the column, HAMLET is eluted

off the column using high salt solution and the complex is further lyophilized into powder and stored in the freezer (at - 20 °C).

HAMLET activity in bacteria

Along with the tumoricidal activity, bacterial killing (bactericidal activity) of pneumococci was also observed in the presence of human milk. This activity was neither mediated by ALA nor by oleic acid (has partial bactericidal activity). HAMLET was later identified as the causing agent of pneumococcal death [112-114]. Other HAMLET-related functions were also identified in bacteria such as growth inhibition (bacteriostatic activity) and altered membrane polarity, integrity and permeability [115, 116].

Methods used to study HAMLET activity in bacteria

Methods used in **paper 1** to study HAMLET's activity in broth grown bacteria (planktonic bacteria) using *in vitro* lab bench settings are shown in **Figure 3**.

Growth inhibition (bacteriostatic activity)

The minimal concentration needed for antibacterial agents (HAMLET, antibiotics or both) to inhibit bacterial growth is known as the minimal inhibitory concentration (MIC). In the MIC assay, the turbidity of an actively growing bacterial culture treated with these agents is detected by measuring the light absorbance over time. The MIC is determined by the lowest concentration at which no bacterial growth (low absorbance indicating no bacterial growth) is detected. The bacteriostatic activity of tested antibacterial agents is illustrated in the form of growth curves, and the fold difference in MIC levels between treated and their corresponding untreated cultures is determined and compared to fold differences of samples treated with other antibacterial agents.

Bacterial killing (bactericidal activity)

Likewise, to determine bacterial viability, the minimal concentration needed for antibacterial agents to induce bacterial killing is known as the minimal bactericidal concentration (MBC). Bacterial cultures treated with antibacterial agents over time are further plated onto blood agar plates and incubated for some time to allow growth of bacterial colonies surviving the treatment. The bactericidal activity of antibacterial agents is then determined by the colony forming unit per ml (CFU/ml) on these plates and compared to their corresponding untreated control. The concentration at which a 3- \log_{10} reduction (or more) of CFU/ml in bacterial cultures

treated with antimicrobial agents as compared to that of non-treated cultures, is the MBC concentration.

Membrane integrity and permeability

To determine the effect of antibacterial agents on the bacterial membrane, the activity of dyes emitting fluorescence (a form of light) were investigated for (1) membrane polarization by which DiBAC₄(3) (Bis-(1,3-Dibarbituric acid)-trimethine oxanol) binding to the bacterial inner membrane emits green fluorescence that indicates membrane depolarization, and (2) membrane integrity by which propidium iodide (PI) binding to bacterial DNA emits red fluorescence that indicates membrane permeabilization and cell death (**Fig. 3 – 4** below). The fluorescence of DiBAC₄(3) and PI in treated cultures is tracked over time, and the fold difference between treated and their corresponding untreated cultures, is determined.

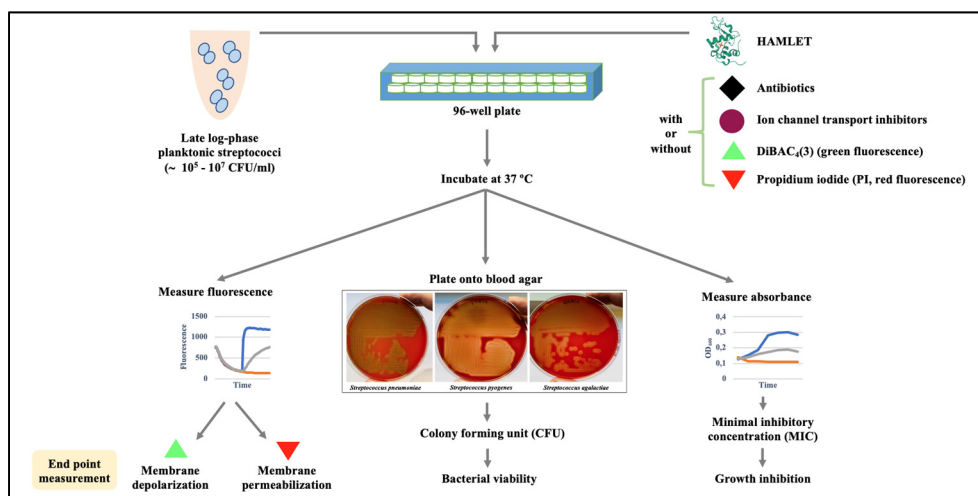


Figure 3. Methods used to study HAMLET's activity in streptococci.

HAMLET sensitivity

In addition to pneumococci, HAMLET-mediated bacterial killing was also observed in other bacterial species such as *Mycobacterium tuberculosis* and the gram-negative *Haemophilus influenzae* [112, 117]. However, this activity is not universal in all bacterial species. Resistance to HAMLET's bactericidal activity has been detected in some bacterial species such as the gram-positive species *Enterococcus faecalis*, *Staphylococcus aureus* and *Listeria monocytogenes*, and the gram-negative species *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Moraxella catarrhalis* [114, 115, 118].

Streptococcus pneumoniae

Earlier studies have shown that broth grown pneumococci (planktonic bacteria), are killed by HAMLET, *in vitro* [112-116]. The mechanisms behind HAMLET's bactericidal activity have mostly been studied in Spn where HAMLET regulates the activity of a set of ion transport channels, carbohydrate metabolic pathways (glucose metabolism) and kinases (enzymes transferring phosphate groups). During HAMLET treatment, membrane depolarization (change in membrane polarity) is maintained through a sodium-dependent influx of calcium ions, which increases the membrane permeability further leading to rupture and cell death (**Fig. 4**) [113, 116, 119]. On the other hand, pneumococci growing in biofilms *in vitro* or colonizing the nasopharynx in mice *in vivo*, are not killed by HAMLET [115].

Using a set of 20 clinical pneumococcal isolates with varying serotypes and antibiotic resistance mechanisms (**paper 1**), we were able to confirm pneumococcal sensitivity to HAMLET and showed that this phenotype is common in all pneumococci. Planktonic growth inhibition and bacterial killing of these isolates were maintained using similar HAMLET concentrations, regardless of the serotype or antibiotic resistance mechanism. During HAMLET treatment, a dose dependent increase in membrane depolarization accompanied with increased permeabilization lead to bacterial death in pneumococci. In accordance with earlier results [116], pneumococcal membrane depolarization and permeabilization were reverted when HAMLET's activity was blocked using ion transport inhibitors such as the calcium transport inhibitor ruthenium red (RuR) and the sodium/calcium exchange inhibitor 3'-4'-dichlorobenzamyl (DCB). These inhibitors also rescued the bacteria from HAMLET-mediated growth inhibition and cell death.

Group A streptococci (GAS)

HAMLET's activity against GAS hasn't been investigated before. In **paper 1**, we were able to show for the first time that GAS bacteria are sensitive to HAMLET using similar HAMLET concentrations as those used to kill pneumococci. Different erythromycin resistant GAS serotypes harboring different resistance mechanisms (ErmB, ErmTR, or MefA and MsrD) were equally sensitive to HAMLET's bacteriostatic and bactericidal activities, regardless of the serotype or antibiotic resistance mechanism. In parallel with Spn, similar mechanisms were involved in HAMLET induced killing of GAS that were also inhibited by RuR or DCB, thus indicating common HAMLET mechanisms in both species (**Fig. 4**).

Group B streptococci (GBS)

In contrast to Spn and GAS, decreased HAMLET sensitivity was observed in clinical GBS isolates harboring unknown or known erythromycin resistance mechanisms (ErmB or ErmTR) (**Paper 1**). In these isolates higher HAMLET concentrations were needed to inhibit growth and kill the bacteria, which confirms earlier studies [114]. As for other streptococci (Spn and GAS), the HAMLET doses

needed to inhibit growth, depolarize and permeabilize bacterial membrane, or kill GBS were the same among GBS isolates and these activities were inhibited using inhibitors targeting calcium and sodium ion transport (**Paper 1** and **Fig. 4** below).

HAMLET potentiated antibiotic activity

Combination treatment with more than one antibiotic is common during treatment of bacterial infections. The idea being that one antibiotic acts as an adjuvant that potentiates the effect of another antibiotic against bacteria [120, 121]. As defined by Liu et al. (2019), “adjuvants are compounds that do not kill bacteria but enhance antibiotic activity by blocking resistance, enhancing intracellular antibiotic accumulation, complementary bactericidal mechanisms, inhibiting signalling and regulatory pathways, or boosting the host response to bacterial infection...” [122].

HAMLET-mediated bactericidal activity is not universal in all bacterial species. While *Staphylococcus aureus* (*S. aureus*) is resistant to HAMLET’s bactericidal activity, membrane depolarization of these species is still induced by HAMLET which further leads to increased membrane permeability and facilitates antibiotic access to the cell. Combination treatment of methicillin resistant *S. aureus* (MRSA) with HAMLET and methicillin (β -lactam antibiotic) reduced the antibiotic MIC and MBC to the sensitivity range [118]. The bacterial viability of biofilms formed by these species *in vitro* as well as nasopharyngeal colonization of mice *in vivo* were also reduced in the presence of HAMLET and methicillin combined, but not by either compound alone [114, 118]. HAMLET-potentiated antibiotic activity has also been observed in *Mycobacterium tuberculosis* (*M. tuberculosis*), by which sub-lethal HAMLET concentrations (mediate membrane depolarization) together with antibiotics eliminated broth grown planktonic bacteria or intracellular bacteria residing within macrophages (immune cells) [117]. Similar effects were also observed in the gram-negative species *Acinetobacter baumannii* and *Moraxella catarrhalis* [115]. Therefore, HAMLET might be used as an antibiotic adjuvant when treating infections caused by these species.

Streptococcus pneumoniae (*Spn*)

As observed in *M. tuberculosis*, combination treatment with sub-lethal HAMLET doses and antibiotics targeting peptidoglycan synthesis (penicillin) or protein production (erythromycin or gentamicin) reduced the antibiotic MIC to sensitivity levels in antibiotic resistant pneumococci [115]. In **paper 1**, we confirmed the previous findings in pneumococci using penicillin or erythromycin, and also showed the efficacy of HAMLET in potentiating the activity of other protein synthesis antibiotics such as clindamycin or kanamycin. In contrast, MIC levels of tetracycline, chloramphenicol, or streptomycin, that also targets protein synthesis, remained the same.

Mechanistically, this could partially be explained by increased binding of the antibiotic to Spn in the presence of HAMLET, as seen for antibiotics (penicillin or gentamicin) coupled with fluorescent dyes. Moreover, blocking calcium transport and kinases rescued pneumococci from the combined bactericidal activity of HAMLET and antibiotics, rendering the bacteria resistant to antibiotics again [115]. Overall, this indicates that HAMLET uses similar mechanisms in pneumococci during treatment alone or in combination with antibiotics (**Fig. 4**).

Moreover, combination treatment of pneumococcal biofilms *in vitro* or infected mouse models *in vivo* also reduced the bacterial viability and inhibited nasopharyngeal colonization [115]. Whether HAMLET uses the same mechanisms in these models as in planktonic bacteria is unknown and needs further investigations.

Group A streptococci (GAS)

The effect of HAMLET and erythromycin combination treatment in erythromycin resistant clinical isolates grown planktonically was investigated for the first time in **paper 1**. Erythromycin resistant GAS isolates harboring ErmB displayed the highest erythromycin MIC levels (2048 µg/ml) and showed the highest fold reduction in erythromycin MIC via HAMLET. On the other hand, erythromycin resistant GAS isolates expressing other resistance mechanisms (ErmTR, or MefA and MsrD) had lower erythromycin MIC levels than ErmB and were reduced to sensitivity ranges during HAMLET and erythromycin combination treatment. Therefore, the mechanisms used by HAMLET during combination treatment in GAS seem to be general mechanisms regardless of the expressed antibiotic resistance protein. Interestingly, as seen in Spn, same HAMLET inhibitory effects were maintained during combination treatment in the presence of inhibitors targeting calcium and sodium ion transport, which further suggests common mechanisms mediated by HAMLET in both species (**Fig. 4**).

In **paper 1**, erythromycin resistant GAS isolates, that are also penicillin sensitive, were subjected to a combination treatment of erythromycin together with HAMLET or penicillin. Interestingly, presence of sub-lethal HAMLET concentrations was more powerful in increasing death and reducing erythromycin MIC levels to the sensitive range in these isolates, as compared to combined treatment with erythromycin and penicillin. Whether these phenotypes and mechanisms are also observed in GAS bacteria grown in biofilms or intracellularly within epithelial cells is unknown and needs to be tested.

Group B streptococci (GBS)

Despite HAMLET resistance in GBS, membrane depolarization and permeabilization still occurred using higher HAMLET concentrations than for other streptococcal species (Spn and GAS). As this resistance phenotype mimics the one observed in *S. aureus*, combination treatment of HAMLET and erythromycin was

tested in erythromycin resistant GBS isolates (**Paper 1**). Similar to GAS isolates, HAMLET and erythromycin combination treatment of GBS isolates carrying ErmB and expressing high erythromycin MIC levels ($> 2048 \mu\text{g/ml}$), resulted in higher fold reduction levels in erythromycin MIC than those harboring ErmTR (also sensitive to combination treatment). Additionally, HAMLET potentiated the effect of erythromycin in two isolates with unknown erythromycin resistance mechanisms and reduced their erythromycin MIC down to sensitivity levels (**Fig. 4**). Similar to GAS, HAMLET potentiated the erythromycin activity more than penicillin in erythromycin resistant GBS isolates regardless of the expressed erythromycin resistance mechanisms. Calcium and sodium transport were also involved in this activity.

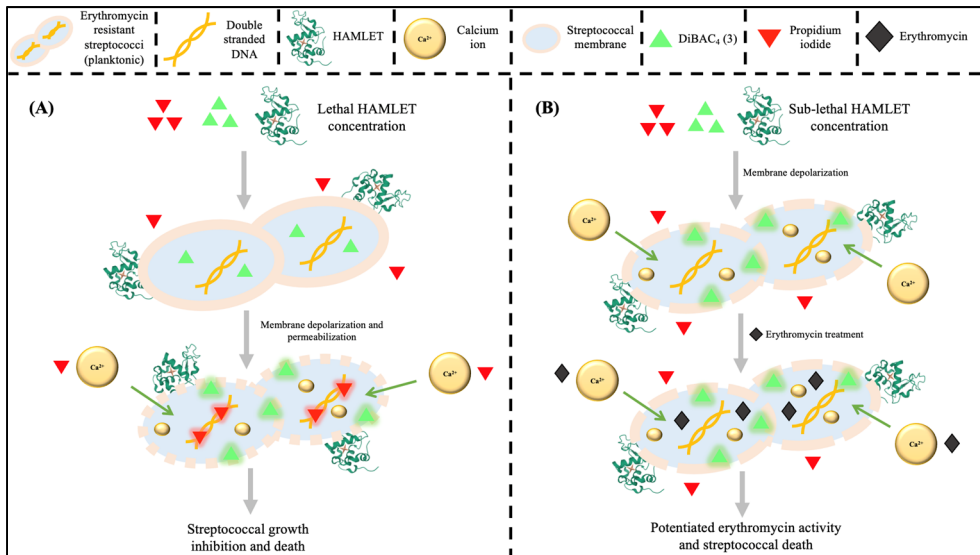


Figure 4. HAMLET mechanisms in erythromycin resistant streptococci during treatment with HAMLET (A) alone, or (B) in combination with erythromycin.

To conclude...

HAMLET-mediated bacteriostatic and bactericidal activities vary between, but not within, bacterial species. Some are sensitive to HAMLET treatment whereas others are more resistant and require higher HAMLET concentrations to be eliminated. Despite resistance, HAMLET exerts depolarization and permeabilization of the bacterial membrane which is needed to potentiate the effect of antibiotics. As a consequence, combination treatment with HAMLET and antibiotics (that bacteria are resistant to) leads to reduced antibiotic MIC levels. The potentiated antibiotic effect was also observed in HAMLET sensitive species using sub-lethal HAMLET

concentrations that mediate membrane depolarization and reduce the antibiotic MIC down to sensitivity levels.

In paper 1, we were able to show that HAMLET sensitivity is not homologous in streptococci. While Spn and GAS showed similar sensitivity phenotypes to HAMLET-mediated bacteriostatic and bactericidal activities, GBS required higher HAMLET concentrations to be killed. These activities were not affected by the serotype or antibiotic resistance mechanism expressed in isolates belonging to the same bacterial type, hence indicating a general HAMLET mechanism among bacteria from the same species. As illustrated in **figure 4**, HAMLET was able to mediate membrane depolarization and permeabilization through sodium and calcium ion transport in all three species and through this mechanism potentiate the effect of antibiotics during combination treatment, thereby reducing the MIC levels of antibiotics that streptococci are resistant to and rendering them sensitive.

These results suggest that HAMLET uses a common mechanism in streptococci. In GAS and GBS, erythromycin activity was potentiated more by HAMLET than by penicillin. HAMLET is therefore suggested as a potential treatment alternative of infections caused by streptococci and other bacterial species (such as *M. tuberculosis* or MRSA). It can be used to directly kill HAMLET sensitive bacteria or indirectly kill both HAMLET sensitive and resistant bacteria by potentiating the effect of antibiotics that these bacteria are resistant to. Therefore, HAMLET has a potential therapeutic role in reducing antibiotic resistance and repurposing antibiotics that were once powerful in treating bacterial infections and that currently lead to therapeutic failure in patients suffering from antibiotic resistant infections.

Chapter 4: Biofilm formation and cellular uptake of *Streptococcus pyogenes*

“Alone we can do so little, together we can do so much” – Helen Keller (1888)

Impermeable and dormant biofilm community development during colonization, is one type of antibiotic avoidance mechanism streptococci (Spn, GAS, or GBS) use to avoid host- or antibiotic-mediated killing [53, 63, 121]. Biofilm formation in model systems mimicking streptococcal colonization is poorly documented and most biofilm studies have been conducted on abiotic surfaces (plastic or glass) that doesn't well represent the human tissue they inhabit. In pneumococci, a well-established biofilm model mimicking nasopharyngeal colonization *in vitro* was developed. In this model, planktonic bacteria are seeded onto pre-fixed epithelial cells in a nutrient-limited medium at a nasopharyngeal temperature of 34 °C [29, 121, 123-125]. Likewise, biofilm formation in GAS has been mostly studied using abiotic surfaces which do not fully represent the physiological niche these species colonize during respiratory infections [61-63]. Recurrent pharyngeal infections and antibiotic treatment failure have been linked to intracellular persistence of GAS, another type of antibiotic avoidance mechanism [63, 71, 72, 74]. Whether GAS colonization and biofilm formation are events preceding this process is not clear and will be investigated in this chapter.

Surface colonization and biofilm formation in GAS

GAS species are sub-divided into 200 serotypes that colonize different niches in the human body [73]. Interestingly, niche-specificity is associated with serotype, so that strains causing pharyngeal infections do not cause skin infections, and vice versa [126-128]. Serotypes associated with pharyngitis commonly express the M proteins 1, 3, 5, 6, 14, 18, 19 or 24, whereas skin infection more often involves strains of M types 2, 49, 57, 59, 60 or 61 [129].

GAS bacteria form long chains when grown planktonically [130]. Upon colonization of the mucosal linings, GAS tend to form complex structured biofilms [29, 51, 61-63]. Using the pneumococcal biofilm model, colonization and biofilm formation of a set of erythromycin resistant clinical isolates (used in **paper 1**) and isolates involved in respiratory infections, were investigated on different epithelial substrata that mimic the upper respiratory tract linings *in vitro*. Developed biofilms were visualized for structure and assessed for maturity, functionality, and composition (**paper 2**). To identify the proteins involved during biofilm formation or needed to form dense and functionally developed biofilms, protein expression profiles of planktonic and biofilm bacteria in four isolates with different biofilm phenotypes were analyzed and compared (**paper 3**). To investigate the uptake and persistence phenotype of biofilm bacteria within respiratory epithelial cells, GAS were grown as biofilms (using the biofilm model in **paper 2 – 3**) and then biofilm bacteria were used to infect live epithelial cells via a live cell infection model (**paper 4**).

Methods used to study GAS biofilms

A set of methods were utilized in **paper 2 - 4** and **chapter 5** (below) to analyze different aspects of GAS biofilms formed on epithelial cells (**Fig. 5**).

Biofilm formation, visualization and assessment

Biofilm formation in GAS was studied on two types of epithelial substrata, the human lung mucoepidermoid carcinoma cell line NCI-H292 (respiratory H292 cells) or the squamous cell carcinoma (SCC) cell line of the facial epidermidis (SCC13 keratinocytes), representing the nasopharynx and oropharynx, respectively [131, 132]. Using the established pneumococcal biofilm model, planktonic bacteria grown to late log-phase were seeded onto pre-fixed epithelial cells (respiratory H292 cells or SCC13 keratinocytes) and grown over time (48 – 72 h) at 34 °C in a nutrient-limited chemically defined medium (CDM), by which fresh media was provided every 12 h. Small scale structures of these biofilms were visualized and magnified images were obtained, via scanning electron microscopy (SEM). To assess biofilm maturity and functionality, developed biofilms were treated with phosphate buffered saline (PBS) or gentamicin (intracellular antibiotic targeting protein synthesis) for 3 h (at 34 °C). After treatment, bacteria were plated onto blood agar plates and incubated until colony formation. The CFU/ml of PBS or gentamicin treated biofilms were determined as a measurement of maturity (indicated by biofilm biomass) or biofilm functionality in escaping the gentamicin activity (indicated by gentamicin sensitivity), respectively.

Extracellular matrix composition

The extracellular matrix (ECM) is an integral biofilm component that supports and stabilizes biofilm structures [133]. To determine if DNA and proteins are ECM components of GAS biofilms, a 3 h enzymatic treatment using proteases that cleave proteins (elastase, proteinase K, papain or trypsin), or DNases that cleave DNA (DNase I or exonuclease I) was applied to developed biofilms at 34 °C. The CFU/ml of bacteria dispersed from biofilms into the surrounding media (supernatant) upon enzyme treatment, were determined and compared to the untreated control.

Adhesion and aggregation of planktonic bacteria

Planktonic bacteria grown in broth were assessed for auto-aggregation (group of bacteria automatically clumping together) over time and adhesion to live epithelial cells by assessing the CFU/ml of plated cellular lysates (remnants of lysed cells).

Protein expression profiles during biofilm formation

To determine the protein expression profiles (proteomes) of pellets containing planktonic or biofilm bacteria, liquid chromatography- mass spectrophotometry (LC – MS/MS) was run. In this method, enzymatic treatment was used to cleave the proteins into peptides that were further separated and identified based on their mass. In each sample, obtained peptide readings were further normalized to a house keeping protein (Gyrase A encoded by *gyrA*) and the protein expression ratio of biofilm bacteria as compared to planktonic bacteria was analyzed.

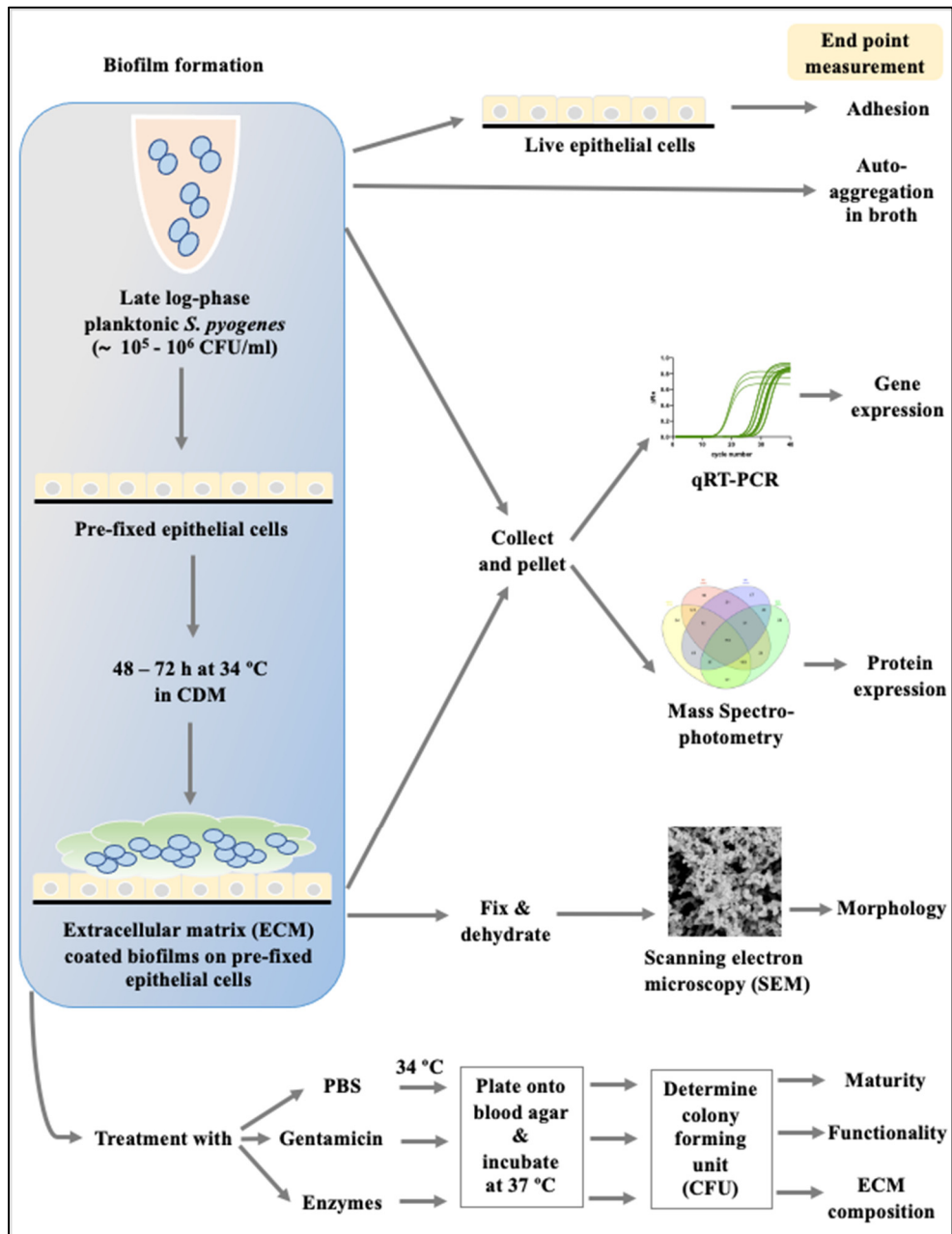


Figure 5. Methods used to study bacterial colonization and biofilm formation in GAS.

Biofilm formation of GAS on epithelial cells

To prove the efficiency of the biofilm model, GAS biofilms were formed on abiotic (plastic or glass) or biotic (epithelial cell substrata) surfaces, and the phenotypes of the developed biofilms were compared (**Paper 2**). The maturity and functionality of biofilms formed on both surfaces were similar, but not identical. Morphologically, biofilms formed on biotic surfaces were denser and more organized than those formed on abiotic surfaces.

Depending on the substrata, phenotypic differences of biofilms formed on different epithelial cells were observed too. Erythromycin resistant isolates (M11, M12, M22, M73, M77 or M89) and isolates commonly causing human respiratory infections (MIT1, M3, M5, M6 or M18) were assessed for biofilm formation over time (48 – 72 h) on respiratory H292 cells or SCC13 keratinocytes. After 72 h, all serotypes formed biofilms with similar, but not identical biomasses. However, differences in biofilm structure, functionality, and biofilm forming kinetics were observed between biofilms formed on these substrata. Overall, GAS colonizing keratinocytes formed mature and functional biofilms faster than those formed on respiratory cells. On the other hand, depending on the serotype, biofilms formed on respiratory epithelial cells exhibited dense biofilm structures containing a network of long bacterial chains coated with ECM or linked by ECM aggregates. The coating ECM contained proteins as well as double stranded DNA, and its presence was essential to maintain biofilm structure and integrity.

Role of auto-aggregation and adhesion during biofilm formation

Auto-aggregation of broth grown planktonic bacteria in GAS is thought to play a role during biofilm formation *in vitro* and microcolony formation *in vivo* [51, 61, 134]. In contrast to these findings, **paper 2** showed no correlation between auto-aggregation, cellular adhesion and biofilm formation in GAS. The MIT1 and M5 isolates that highly auto aggregated in broth, adhered in high numbers to cells, but formed biofilms with different functionalities. On the other hand, the M3 isolate that had a low auto-aggregation ability and adhered less to cells, was still able to develop mature and functional biofilms. Therefore, other factors, not related to auto-aggregation and cell adhesion, play a role during biofilm formation in GAS.

Protein regulation during biofilm formation

In pneumococci, the lifestyle of biofilm bacteria differs from that of planktonic bacteria, by which re-arrangement of the metabolic activity, reduced energy production as well as down-regulated expression of proteins involved in virulence or capsule production, is made when planktonic bacteria settle as biofilms [53]. To determine if planktonic GAS bacteria behave the same as pneumococcal bacteria during colonization, proteomes of biofilm bacteria formed by the GAS isolates MIT1, M3, M5, or M18, were analyzed and compared to the proteomes of their corresponding planktonic bacteria. Along with that, proteins needed during biofilm

formation in GAS were determined (**paper 3**). The ability to form biofilms in GAS wasn't homologous in all serotypes, some formed dense and functionally mature biofilms whereas others formed thinner and less functional biofilms. To investigate the protein expression profiles of these biofilms, we selected two strains from each biofilm phenotype such as the M5 and M18 strains forming dense biofilms, and the M1T1 and M3 strains forming thin biofilms. The protein expression pattern of biofilm bacteria isolated from dense or thin biofilms differed. More than half the proteome was up-regulated in bacteria isolated from thin biofilms, whereas a smaller part of the proteome in dense biofilms showed regulation where most proteins were down-regulated.

On the other hand, all four serotypes commonly regulated a set of proteins in their biofilms that are potential and interesting candidates involved in biofilm formation. In general, biofilm bacteria commonly down-regulated 6 proteins involved in arginine metabolism (amino acid metabolism) and other process (methylation, protein catabolism, or amino acid and vitamin metabolism). In contrast, a network of 75 proteins involved in transport, DNA/RNA metabolism, carbohydrate metabolism, protein synthesis (translation) as well as transcriptional regulation, were up-regulated. Transcriptional regulators are proteins that control the expression of other proteins. The expression of a set of transcriptional regulators was induced in biofilm bacteria such as the CovR (also known as CsrR) response regulator, RegR regulator, and the GntR family regulator, all correlated to virulence in streptococci [135-137]. The CovR (or CsrR) response regulator is part of the two-component CovRS system (also known as the CsrRS system) that has been shown to play a role during biofilm formation and invasion in GAS, and is involved in regulating the expression of 15% GAS proteins among which gene expression of virulence factors is suppressed (down-regulated) [62, 137-139].

Uptake and persistence of biofilm bacteria in epithelial cells

Another form of antibiotic avoidance mechanism in GAS, is the escape of host- or antibiotic-mediated bacterial killing via intracellular residence. This mechanism is thought to be the reason behind recurrent pharyngeal infections caused by GAS, where intracellular bacteria, after completed antibiotic treatment, re-emerge and cause recurrent infections [72, 74].

Similar to the T3SS in gram-negative species, *S. typhimurium* and *S. flexneri* (mentioned in **chapter 1**), a functional equivalent of T3SS termed cytolysin-mediated translocation (CMT) has been detected in the gram-positive bacterium GAS [41]. Uptake of GAS into epithelial cells via actin rearrangements and membrane ruffles have been documented, indicating uptake via trigger mechanisms,

but it is not clear whether this is mediated via CMT [140, 141]. Similar to *L. monocytogenes*, uptake of GAS bacteria into epithelial cells via zipper mechanisms has been shown before [31, 142]. Depending on the strain, clathrin-mediated uptake of individual or aggregates of planktonic bacteria into epithelial cells that are detected in lysosomes, autophagosomes or another uncharacterized intracellular environment have been documented [33, 142-144]. However, it is not known whether intracellular GAS bacteria behave in a similar way as *L. monocytogenes* and escape vacuoles into the cytoplasm to maintain intracellular persistence and use actin for motility. Overall, for unknown reasons, uptake mechanisms in GAS vary between strains where some use the trigger mechanism whereas others use the zipper mechanism [33, 140, 142, 145, 146]. Uptake of biofilm bacteria have also been documented in these species, but whether biofilm bacteria are taken up as individual bacteria by zipper or trigger mechanisms or as aggregates via invasomes is not known [29]. Besides, the correlation between colonization, biofilm formation and cellular uptake is not clear.

To address this, we used a live cell infection model mimicking respiratory infections *in vitro* (**paper 4**). In this model, GAS bacteria (planktonic or biofilm bacteria) were seeded onto respiratory epithelial cells for certain periods of time and the association, internalization, and persistence rates of GAS bacteria, were investigated. Along with that, cellular uptake pathways (endocytic pathways) utilized by bacteria during uptake were determined, intracellular bacteria were localized, and the consequent cellular responses were studied.

Tools used to study intracellular persistence of GAS

The live cell infection model along with other methods were used to study uptake and persistence of GAS within respiratory epithelial cells (**Fig. 6**)

Live cell infection model and consequent cellular responses

In this model, GAS bacteria grown in broth (planktonic) or biofilms were used to infect live respiratory epithelial cells. After infection, cells were either left untreated or treated with antibiotics (penicillin and gentamicin) to eliminate extracellular bacteria. Cell lysates of antibiotic treated samples were used to determine the CFU/ml of intracellular bacteria. Subtracting this value from the lysate CFU/ml of non-treated samples, the amounts of bacteria associating to the outside of epithelial cells were determined (indicated by a red star in **Fig. 6**). Supernatants of untreated samples were used to determine bacterial growth during infection, whereas supernatants of antibiotic treated samples were analyzed for consequent cellular responses to bacterial invasion over time (4 – 44 h). These responses include the release of inflammatory mediators such as chemokines (Interleukin- 8; IL-8) that attract nearby immune cells, and cytokines (IL-1 β , IL-6, IL-10, IL-12, or Tumor Necrosis Factor α ; TNF- α) that signal immune cells *in vivo* and modulate their

activity. In parallel, bacterial toxicity to epithelial cells (cytotoxicity) was determined by detecting the Lactate Dehydrogenase (LDH) levels in supernatants of infected cells. The rationale behind this is to determine whether reduced bacterial persistence (reduced lysate CFU/ml) is (1) due to cell-mediated killing of intracellular bacteria, or (2) due to bacterial cytotoxicity in epithelial cells further leading to cell lysis (determined by elevated LDH levels) and release of intracellular bacteria into the surrounding medium that are finally killed by antibiotics present in the medium.

Identifying cellular uptake pathways

Using the same model, respiratory H292 cells untreated or pre-treated with inhibitors targeting proteins involved in cellular uptake pathways (actin, dynamin, microtubulin, lipid raft- or clathrin-mediated uptake), were infected with GAS bacteria. After infection, the role of cellular uptake pathways during internalization were determined in lysate CFU/ml of inhibitor treated cells as compared to non-treated cells.

Bacterial localization

To determine the bacterial location within epithelial cells, we infected live epithelial cells with fluorescent GAS bacteria emitting green color when exposed to light. Fluorescent bacteria binding to the cell surface extracellularly or residing intracellularly were visualized and localized using fluorescence microscopy that detect the emitted color from bacteria. To differentiate between extracellular and intracellular bacteria, we used fluorescent anti-GAS antibodies that bind to targets exposed on the surface of extracellular bacteria, but not intracellular bacteria, thereby emitting red color. Additionally, to specify the bacterial location onto or within cells, cellular structures such as the DNA containing nucleus and the cytoskeleton protein actin, were counterstained with fluorescent dyes emitting blue and orange color, respectively.

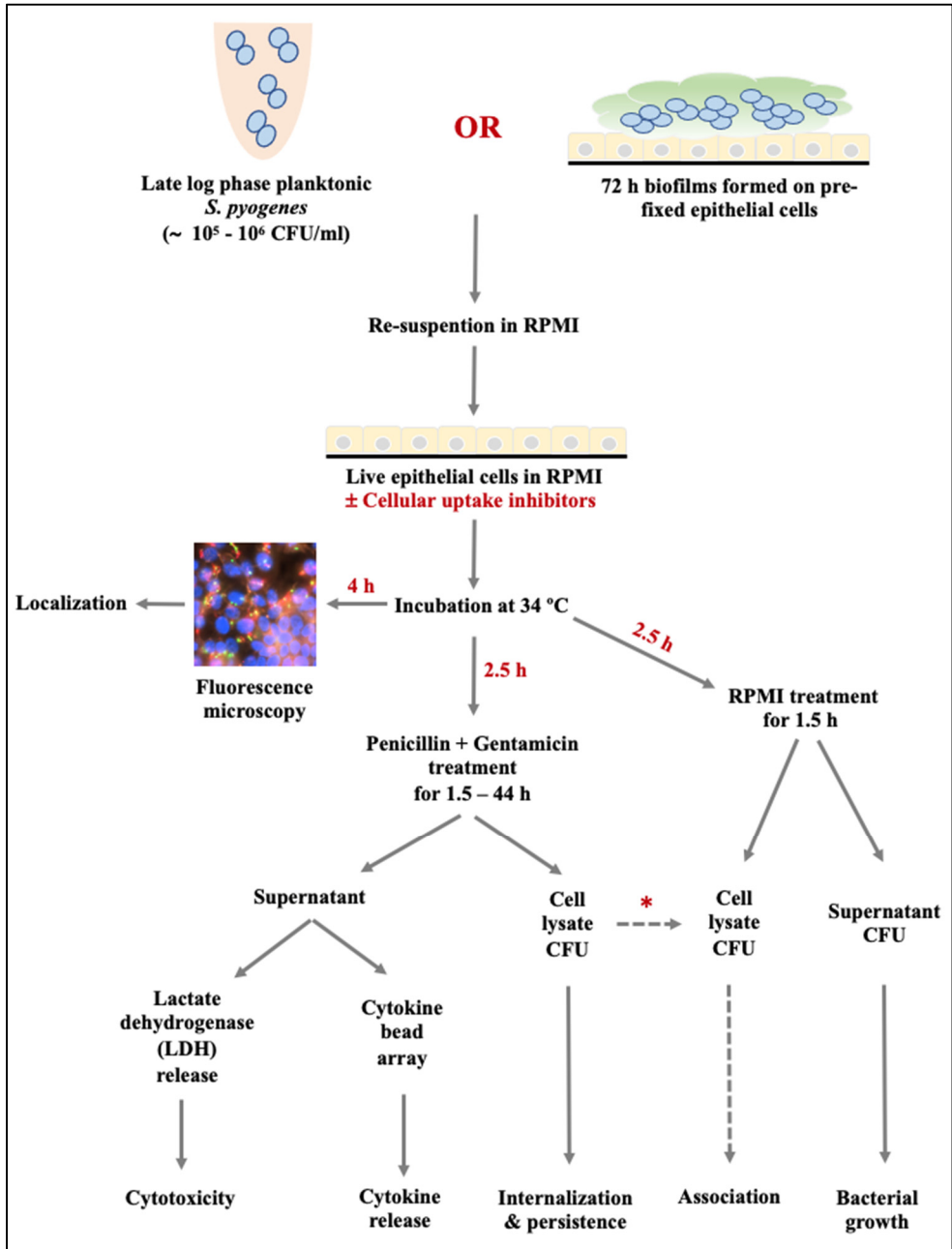


Figure 6. Intracellular invasion studied using the live cell infection model. * Association CFU/ml are indirectly determined by subtracting lysates of antibiotic treated and untreated cells.

Uptake and persistence of GAS within epithelial cells

Confirming earlier studies [29], biofilm bacteria of the M3 isolate internalized in higher numbers, persisted longer (more than 44 h), and were killed slower than planktonic bacteria, despite similar association levels (**paper 4**). However, the differences in uptake and persistence levels between both bacterial forms were not common in all isolates. While uptake levels and persistence duration (more than 44 h) of biofilm bacteria were similar between isolates, these aspects varied between planktonic bacteria. Similar uptake and persistence levels of biofilm bacteria among GAS isolates could possibly be explained by similar uptake mechanisms and regulation of proteins involved in these processes once bacteria settle into biofilms. Planktonic bacteria taken up and persisting similarly as biofilm bacteria could have similar uptake mechanisms and protein regulation which, however, might differ in those taken up less and persisting shorter. Depending on the isolate, similar or different killing rates of intracellular bacteria in both bacterial forms were observed, which suggest similar or different uptake mechanisms and intracellular trafficking of these bacteria. Depending on where the bacteria end up within the cell, their survival rate is affected by which intracellular bacteria will either have an easier or more difficult time surviving cell-mediated killing. Additionally, no correlation was found between the association and internalization levels of GAS bacteria which suggests that bacterial uptake (internalization) is not associated with bacterial binding to the surface of epithelial cells.

Microscopically, small clumps or individual planktonic bacteria were found in the cytoplasm of respiratory epithelial cells, whereas larger aggregates of biofilm bacteria were present in close proximity to the perinuclear area and highly colocalized with actin. Bacterial aggregates localizing in the perinuclear area can be correlated to the previously observed phenotype of GAS localization in lysosomes or autophagosomes, possibly indicating uptake of biofilm bacteria into these structures [33]. Overall, these results suggest that planktonic and biofilm bacteria use different uptake pathways and intracellular trafficking where they end up in different structures within cells that in turn lead to differences in the persistence and killing rates.

Endocytic pathways involved in bacterial uptake

General endocytic mechanisms like actin polymerization or dynamin function were involved during uptake of planktonic and biofilm bacteria of GAS into epithelial cells. However, microtubulin or lipid raft mediated uptake did not seem to be involved. Along with earlier findings [33, 142], planktonic bacteria utilized a more specific uptake mechanism via clathrin by which blocking this protein partially blocked planktonic uptake, however this was not seen in biofilm bacteria. Uptake of bacterial aggregates in *B. henselae* into invasomes via actin, talin-1 and β 1-

integrins has been documented [34, 35]. As large aggregates of biofilm bacteria in GAS were detected in epithelial cells, it could possibly be that biofilm bacteria utilize the same mechanism as *B. henselae* to enter cells. To be able to address that, further investigations using inhibitors targeting talin-1 or β 1-integrins, are needed. The trigger mechanism could also be a possible involved mechanism which can be explained by actin polymerization and co-localization as well as the non-essential bacterial-cell contact (association) during bacterial uptake. To address this, GAS infected cells should be investigated microscopically for membrane ruffles and actin polymerization using fluorescent actin and membrane dyes. If that is the case, then the role of CMT should be identified in this uptake mechanism, to see if GAS biofilm bacteria use the same uptake mechanism as *S. typhimurium* or *S. flexneri*.

Epithelial cell responses to GAS infection

IL-8 release from epithelial cells has been shown to be regulated by IL-6 in the form of autocrine signalling (molecules inducing signalling in the same cells that produce them) *in vivo*, which further leads to neutrophil (immune cell) recruitment from the bloodstream [147, 148]. In response to infection by the M3 isolate, epithelial cells released increasing levels of IL-6 and IL-8 over time. This, therefore, supports the classical recruitment of neutrophils during respiratory GAS infections *in vivo*. On the other hand, inflammatory mediators like IL-1 β , IL-10, IL-12, or TNF- α were not detected in the supernatant of infected cells.

To conclude...

In **paper 2 - 3**, we used a well-established biofilm model *in vitro* and showed that GAS form dense, mature and functional biofilms on epithelial substrata mimicking the respiratory niche of GAS. These biofilms were more developed than those formed on abiotic surfaces. Differences within substrata were also observed, biofilms formed on keratinocytes developed and functionally matured faster than those formed on respiratory epithelial cells. When colonizing the respiratory epithelium, GAS rearrange its metabolic activity and repress the expression of certain regulators to form these biofilms. However, this was not dependent on the bacterial ability to auto aggregate or adhere to cells, instead the biofilm forming ability of each strain was the deciding factor. The morphology, the maturity and the functionality of biofilms weren't homologous between GAS isolates. Some isolates formed dense and functionally mature biofilms that down-regulated most of its regulated proteins, whereas others formed thin and less functional biofilms that might be due to highly up-regulated protein expression in their proteomes. Structurally, depending on the underlying substrata, GAS biofilms of the M3 isolate contained bacterial chains that were either linked or coated by ECM containing

proteins and DNA. This matrix preserves the structure and integrity of biofilms and protects the biofilm bacteria from external threats such as antimicrobial agents.

In **paper 4**, using *in vitro* settings, we showed that bacterial association is not essential during uptake into respiratory epithelial cells. We confirmed earlier studies and showed a partial role of clathrin during uptake of small clumps or individual planktonic bacteria into epithelial cells that were localized in the cytoplasm, suggesting a similar uptake mechanism (clathrin-mediated uptake) as *L. monocytogenes*. On the other hand, internalization of large aggregates of biofilm bacteria localizing in the perinuclear area and persisting intracellularly over 44 h was observed. The perinuclear localization could be correlated to uptake via lysosomes or autophagosomes. In all GAS isolates, uptake numbers and persistence duration of biofilm bacteria were the same indicating common uptake mechanisms in biofilm bacteria, whereas those of planktonic bacteria varied. Actin polymerization and dynamin functionality were involved in the uptake process of both bacterial phenotypes, however the specific mechanisms behind biofilm bacterial uptake remain mysterious. The intracellular persistence of both bacterial forms was non-toxic to epithelial cells, however IL-6 and IL-8 release was detected over time as a response to GAS infection, which correlates to the classical neutrophil recruitment during GAS infections *in vivo*.

Overall, these findings indicate the presence of antimicrobial avoidance mechanisms in GAS that support bacterial survival during GAS infections and are either mediated by the rigid biofilm structure and dormant phenotype during colonization, or the intracellular persistence of biofilm bacteria within respiratory epithelial cells. In terms of pathogenesis, intracellular localization and persistence of antibiotic insensitive biofilm bacteria could possibly explain the reason behind treatment failures during recurrent pharyngeal infections caused by GAS. Overall, these findings help in better understanding GAS pathogenesis and aid in finding better treatment alternatives for respiratory infections caused by these species.

Chapter 5: Factors utilized during GAS pathogenesis of the respiratory epithelium

“If you know your enemies and know yourself, you will not be imperiled in a hundred battles; if you do not know your enemies but do know yourself, you will win one and lose one; if you do not know your enemies nor yourself, you will be imperiled in every single battle.” – Sun Tzu (5th century BC)

Understanding the process of GAS mediated respiratory infections and the factors commonly utilized by this pathogen during pathogenesis, will help in improving treatments of respiratory infections and define potential targets involved in GAS infections that could be of therapeutic interest. As mentioned in **chapter 4** and shown in **paper 3**, GAS biofilms commonly up-regulate the protein expression of the two-component CovRS system, which is a global regulator that controls the expression of 15% of the GAS genome and suppress the expression of genes/operons (group of related genes) encoding the M protein (Emm), hyaluronan synthase involved in capsular biosynthesis (HasA), streptococcal pyrogenic exotoxin B (SpeB), streptolysin O (SLO), NAD-glycohydrolase (NADase), streptolysin S (SLS), and others [149, 150]. GAS strains lacking CovRS (mutants) were much more virulent in mouse infection models by which mutants isolated from infected tissues exhibited high gene expression levels of the virulence related proteins, SpeB and SLS [137-139]. Additionally, the CovRS mutant failed to develop biofilms due to increased expression levels of virulence factors which explains the down-regulated gene expression of the M protein, hyaluronic acid capsule, SpeB, SLS, and NADase during biofilm formation in GAS strains harboring this regulator [29, 62]. Overall, up-regulation of CovRS is essential during biofilm formation in which it represses the gene expression of several virulence factors that in turn repress GAS virulence *in vivo*. However, the exact role of each of these proteins during colonization and biofilm formation (on biological surfaces), or uptake and persistence of biofilm bacteria, is not clear. Therefore, strains lacking or expressing the M protein, hyaluronic acid capsule, SpeB, SLO, NADase or SLS, were investigated for these aspects (**paper 2 - 4**).

Strains and methodology used

To address the role of virulence factors during colonization, biofilm formation, and pathogenesis of GAS, the same methods described in **chapter 4** (shown in **Fig. 5** and **Fig. 6**) were applied to strains lacking (mutants, denoted as Δ) or expressing (wild type, denoted as WT) these factors (**Fig. 7 – 8**). Along with the proteomic analysis in **paper 3**, gene expression analysis of virulence factors in GAS biofilms formed on biological surfaces, was investigated.

Gene expression

To assess the gene expression profiles in planktonic or biofilm bacteria, first cDNA is synthesized from isolated bacterial RNA (reverse transcription) in a polymerase chain reaction (PCR) machine. The obtained cDNA is amplified into several copy numbers that are quantified and tracked via SYBR green, a DNA binding dye, through quantitative real time PCR (qRT-PCR). The obtained reads for each gene of interest were then normalized to a house keeping gene (*gyrA*) and the fold difference in gene expression in biofilm bacteria as compared to planktonic bacteria was determined.

Strains used to investigate the virulence factors involved in GAS pathogenesis

The role of M protein during biofilm formation and invasion was investigated in a set of clinical isolates harboring different M protein types (serotypes), or strains expressing or lacking the M protein gene (Δemm). Furthermore, the role of the hyaluronic acid capsule was investigated in strains expressing varying levels of capsule or lacking the expression of the capsular biosynthesis protein termed HasA ($\Delta hasA$). Moreover, the role of other virulence factors was investigated in M3 strains lacking the gene expression of SpeB ($\Delta speB$), SLS ($\Delta sagA$), SLO (Δslo), or NADase (Δnga).

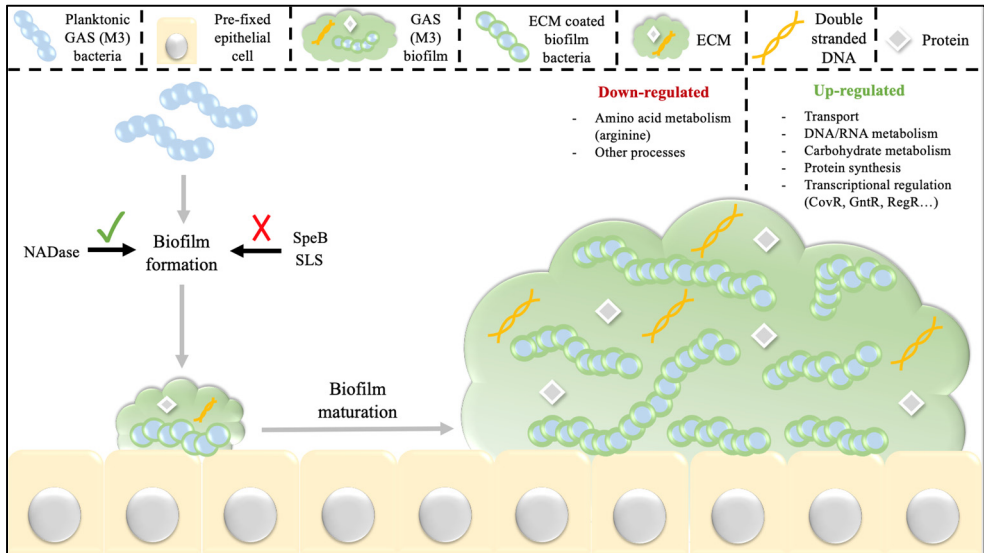


Figure 7. Virulence factors involved during biofilm formation of M3 GAS on pre-fixed respiratory epithelial cells along with the protein expression (down-regulated or up-regulated) profiles of biofilm bacteria as compared to the corresponding planktonic bacteria .

Surface M protein

The surface M protein is known for its role as a cell adhesion factor and have been implicated in GAS colonization and biofilm formation [62, 134, 151-154]. This protein is encoded by the *emm* gene whose expression is regulated by the global regulator Mga (*mga*). GAS strains lacking *mga* has a decreased expression of the M protein as well as reduced biofilm formation on abiotic surfaces [134]. Additionally, down-regulated expression of *emm* has been detected in mature M3 biofilms formed on biological surfaces *in vitro*, which suggests an inhibitory role of the M3 protein during later stages of biofilm development [29]. The M3 protein has been shown to be required for optimal internalization of planktonic GAS bacteria into epithelial cells [155]. Similarly, the M1 protein has been shown to be required during uptake and persistence of GAS bacteria in endothelial or immune cells (macrophages) [156, 157]. On the other hand, the M5 or M18 protein controlled bacterial adhesion to epithelial cells and inhibited bacterial uptake by human blood cells [154]. However, the precise role of these M proteins during biofilm formation on biological surfaces and uptake of biofilm bacteria into epithelial cells is unknown

Up-regulated protein expression of Mga was accompanied with up-regulated levels of the M3 protein in biofilms formed by the M3 isolate. However, this was not the case in other serotypes, by which Mga protein expression was slightly or significantly up-regulated, whereas the protein expression of M1, M5, or M18, was

down-regulated. Gene expression analyses of Mga and M protein in these isolates was not successful and needs to be further optimized before drawing conclusions regarding the correlation between Mga and M protein expression profiles (**not shown in paper 3**).

As discussed earlier in **chapter 4** (also shown in **paper 2 - 4**), serotypes expressing different M proteins formed biofilms with similar biomasses, but with varying biofilm forming kinetics and functions. Biofilm bacteria of these isolates internalized and persisted equally long within epithelial cells, however a difference in the uptake and persistence of the corresponding planktonic bacteria was observed.

The role of the M protein during biofilm formation, uptake, and persistence within epithelial cells, was more directly addressed in biofilms formed by different GAS serotypes expressing (M1T1, M3 or M5) or lacking ($\Delta emm1$, $\Delta emm3$, or $\Delta emm5$) the M protein gene (**paper 2 - 4**). Due to the down-regulated protein expression levels in biofilm bacteria, the M1 or M5 protein did not play any significant role during biofilm formation, uptake, or persistence. Although M3 is known for its adhesion properties, however its absence did not affect bacterial adhesion or biofilm formation onto epithelial cells. Thus, indicating the presence of other factors needed during bacterial adhesion. On the other hand, the M3 protein was needed to mediate a compact biofilm structure, as well as intracellular persistence of planktonic or biofilm bacteria within epithelial cells (**Fig. 8**), thereby confirming earlier results that correlates this protein to GAS uptake [155].

Overall, *in vitro*, these findings indicate a possible role of the M3 protein during GAS pathogenesis. The role of the M1 or M5 protein during biofilm formation is unclear and needs further investigation. On the other hand, presence or absence of these proteins did not affect the uptake or persistence of GAS bacteria into epithelial cells, which indicates the presence of other proteins needed during these processes. Whether this is the case *in vivo*, animal models infected with these mutants and investigated for pathogenesis should be performed to confirm these observations.

Hyaluronic acid capsule

The hyaluronic acid capsule serves as a protecting shield for GAS during infection and capsule expression play an enhancing role in mediating persistent pharyngeal colonization and phagocytosis escape in animal models [151, 158-161]. *In vitro*, interaction of capsule with epithelial cell receptors like CD44 allows cytoskeletal rearrangements and membrane ruffle formation which suggests a role of capsule during uptake via trigger mechanisms [141]. The role of capsule during biofilm formation is not clear, while capsule expression is down-regulated in biofilms formed on pre-fixed epithelial cells, up-regulated capsule expression has been observed in infected zebrafish models [29, 62]. Therefore, the role of capsule during

biofilm formation and intracellular persistence within epithelial cells was investigated in **paper 2 – 4**.

Interestingly, a correlation between capsule expression and biofilm formation was observed in GAS, where highly encapsulated strains (M5 or M18) formed dense and functionally developed biofilms whereas strains exhibiting lower capsule amounts (M1T1 or M3) formed thinner and less-functional biofilms (**paper 2**). However, the capsule amount within dense or thin biofilm structures remain unknown. Strains forming dense biofilms slightly up-regulated the gene expression of the CovRS regulator whereas down-regulated expression levels were seen in those forming thin biofilms. Hypothetically, reduced biofilm forming ability in strains forming thin biofilms could be correlated to the reduced CovRS suppression of *hasA*, consequently leading to increased gene and protein expression levels of the capsular biosynthesis protein HasA. On the other hand, those forming dense biofilms successfully suppressed the gene and protein expression of HasA via up-regulated CovRS expression (**paper 3**). However, the precise role of capsule during biofilm formation is not clear, since non-encapsulated GAS mutants ($\Delta hasA$) lacking capsular production formed dense and well-developed biofilms that were structurally different from WT biofilms (**paper 2**).

Capsule expression has been shown to block GAS uptake into epithelial cells [33, 162]. In parallel with these findings, planktonic bacteria of strains expressing low capsule amounts were internalized more than those of highly encapsulated strains. On the other hand, biofilm bacteria of strains expressing varying capsule amounts internalized and persisted equally well within epithelial cells. Hypothetically, this can be explained by rearranged protein and gene expression of HasA in GAS biofilms where highly encapsulated strains down-regulate their capsule expression whereas those covered by lower amounts of capsule up-regulate their capsule expression, finally leading to equal production of capsule amounts within biofilms (**paper 3 - 4**). Confirming the inhibitory role of capsule during uptake, non-encapsulated GAS mutants ($\Delta hasA$) internalized more than their encapsulated WT strains. On the other hand, intracellular biofilm bacteria of $\Delta hasA$ persisted less than those of WT, indicating a role of capsule in mediating prolonged intracellular persistence of biofilm bacteria (**Fig. 8**). Therefore, it could possibly be that intracellular biofilm bacteria express certain capsule amounts to maintain persistence and escape host-mediated killing. However, further investigations are needed to confirm this hypothesis.

Overall, the hyaluronic acid capsule seems to inhibit colonization and biofilm formation in GAS, therefore, suppressed capsule expression via CovRS is needed to form dense and well-developed biofilms. Along with earlier studies, capsular presence inhibits endocytosis, however a certain level of capsule expression might be needed to mediate prolonged intracellular persistence of biofilm bacteria.

The scarlet fever toxin SpeB

One of the earliest identified GAS secreted protein and also the most studied proteinases of any pathogen, is the streptococcal pyrogenic exotoxin B (SpeB, also known as the scarlet fever toxin or erythrogenic toxin) encoded by *speB* [163, 164]. This toxin is a cysteine protease that has been shown to inhibit biofilm formation *in vitro*, and therefore down-regulated expression of *speB* during biofilm formation has been observed in GAS [29, 64, 165, 166]. *In vivo*, SpeB expression, controlled by a Srv regulator, mediates biofilm formation, dissemination, and invasion [64, 166]. Similar to capsule, SpeB inhibits bacterial uptake into epithelial cells *in vitro* [162].

Confirming earlier studies, M3 strain lacking SpeB (Δ *speB*) formed dense and well-developed biofilms indicating an inhibitory effect of SpeB on biofilm formation (**Fig. 7**). The morphology, the functionality and the proteomic profiles of biofilms formed by Δ *speB* resembled the dense and functionally developed biofilms formed by M5 or M18 (**Paper 2-3**). Biofilm bacteria of Δ *speB*, M5, and M18, all down-regulated proteins involved in carbohydrate metabolism, cell wall biogenesis, translation and transport, that were, however, up-regulated in thin biofilms formed by the corresponding WT strain (M3). Suppressed *speB* expression, probably via CovRS, was detected in biofilms formed by all tested GAS strains (MIT1, M3, M5 or M18), however the protein expression levels varied between strains forming dense (M5 or M18) or thin biofilms (MIT1, not detected in M3).

Therefore, down-regulated expression (gene and protein expression) of SpeB, along with reduced expression of proteins involved in carbohydrate metabolism, cell wall biogenesis, translation, and transport, are associated with the dense and well-developed biofilm phenotypes (**paper 2 - 3**). In terms of cellular invasion, and in contrast to earlier studies, SpeB did not affect the uptake of GAS bacteria into live epithelial cells, however SpeB expression was needed to mediate prolonged intracellular persistence of biofilm bacteria (**paper 4 and Fig. 8**).

Streptolysin O and its co-toxin NADase

Another GAS secreted protein is the hemolysin streptolysin O (SLO) that has the ability to lyse red blood cells [167]. Lysis of keratinocytes and certain immune cells due to the cytolytic activity of this enzyme has been observed *in vitro*, hence defining it as a toxin. *In vivo*, phagocytosis protection and enhanced virulence of non-encapsulated GAS strains is mediated by SLO in sepsis infection models (but not soft tissue infection models) [168]. Similar to the LLO function in *L. monocytogenes*, SLO is also a CDC that forms pores in membranes when being injected by the CMT in GAS, thereby facilitating entrance of its co-expressed

NADase toxin [31, 36, 41]. When present outside cells, *in vitro*, these toxins act synergistically and exert cytotoxic injuries in epithelial cells [41, 168, 169]. SLO has been shown to play a role during GAS uptake into epithelial cells via clathrin-mediated endocytosis (zipper mechanism) which resembles the uptake mechanism of *L. monocytogenes* [41]. Once inside cells, release of both SLO and NADase by intracellular bacteria inhibits cell-mediated bacterial killing and protects intracellular residence of GAS bacteria [170]. *In vitro*, *slo* expression in biofilms formed on epithelial surfaces was unchanged, whereas down-regulated expression levels of *nga* was detected in biofilms formed on abiotic surfaces [29, 62]. However, the exact role of these toxins during colonization, biofilm formation and uptake into epithelial cells, is unclear.

Despite down-regulated protein levels of SLO and NADase in biofilm bacteria of most serotypes, biofilms formed by strains lacking SLO (Δslo) or NADase (Δnga) expression exhibited inverse phenotypes. As compared to biofilms formed by the corresponding WT strain, denser and highly functional biofilms were formed by Δslo , which suggests SLO mediated inhibition of biofilm formation when expressed in GAS (**paper 2**). On the other hand, thinner and less functional biofilms were formed by Δnga whose proteome (mostly up-regulated) closely resembled that of thin biofilms formed by its corresponding WT (M3) or MIT1 strain. However, down-regulated protein and gene expression of NADase was also observed in strains forming dense and well-developed biofilms (**paper 2 - 3**). Therefore, the exact role of NADase during biofilm formation in GAS is not clear. One possible hypothesis could be that NADase is only needed during biofilm formation in M3, but not in other serotypes (**Fig. 7**).

On the other hand, no role of SLO or NADase was detected during GAS uptake, still NADase was needed to mediate prolonged intracellular persistence of biofilm bacteria (**Fig. 8**). In the absence of SLO, planktonic and biofilm bacteria were found in the perinuclear area, whereas planktonic bacteria expressing the toxin were detected in other cellular areas. On the other hand, SLO had no effect on the persistence and killing rates of intracellular bacteria, suggesting that this toxin might have a different intracellular function than LLO (**paper 4**). Interestingly, SLO and NADase, or SLO and capsule, combined, inhibited biofilm formation and uptake of GAS bacteria into epithelial cells, thus suggesting the involvement of additional virulence factors whose expression is affected by the absence of SLO alone, NADase alone, SLO and NADase, or SLO and capsule.

Overall, in the M3 strain at least, these results suggest an essential role of NADase to maintain biofilm formation and prolonged persistence of intracellular biofilm bacteria. On the other hand, SLO inhibited biofilm formation, but was needed for intracellular trafficking of GAS bacteria within epithelial cells.

Streptolysin S

Another secreted hemolysin is the *sagA* encoded Streptolysin S (SLS) whose enzymatic activity is responsible for the beta hemolytic zones formed around GAS colonies on blood agar plates (**Fig. 1**) [167]. Epithelial cell injury, but not lysis, has been shown to be mediated by the toxic activity of GAS secreted SLS [168]. Moreover, down-regulated *sagA* expression was detected in biofilm bacteria formed on epithelial cells, however the precise role of SLS during biofilm formation and uptake into epithelial cells is unknown [29].

Similar to SLO, biofilm formation was inhibited by SLS which could be explained by the commonly reduced *sagA* expression in GAS biofilm bacteria. In contrast, SLS did not play any significant role during cellular uptake of GAS bacteria. However, it was only needed for optimal persistence of planktonic bacteria (**paper 2 - 4**).

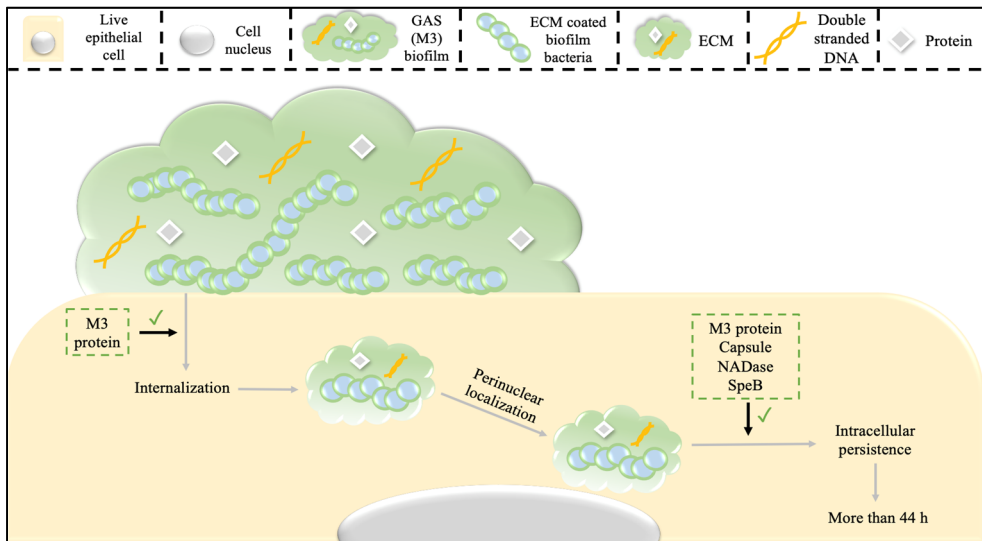


Figure 8. Virulence factors involved during uptake and persistence of GAS biofilm bacteria within respiratory epithelial cells.

To conclude...

Our *in vitro* studies show that the protein expression of the global regulator CovRS is commonly up-regulated in GAS biofilms formed on epithelial cells surfaces. In contrast, its gene expression varied among serotypes which could possibly explain the different biofilm phenotypes (dense or thin biofilms) as a consequence of

varying capsule, and to some extent SpeB, expression. The precise role of these factors and other virulence related factors (M protein, SLO, NADase, or SLS) during GAS pathogenesis, was investigated in strains expressing or lacking the genes encoding these proteins (**Fig. 7 – 8**).

Our obtained findings suggest that GAS bacteria, particularly the M3 strain, express NADase and a certain level of capsule to mediate GAS colonization of pre-fixed epithelial cells and settle into biofilms (**Fig. 7**). To prevent the inhibitory effect of SpeB and SLS on biofilm formation, GAS bacteria down-regulate the gene expression of these toxins. These findings confirm previous results that show an enhancing role of capsule during colonization as well as an inhibitory role of SpeB during biofilm formation.

For the first time, we were able to show that biofilm bacteria, along with planktonic bacteria, use the M3 protein to enter epithelial cells which confirms earlier results indicating a role of this adhesin during uptake of GAS bacteria (**Fig. 8**). Once inside cells, intracellular biofilm bacteria re-arrange protein expression of some virulence factors by which capsule, NADase, SpeB, along with the M3 protein, are needed to maintain prolonged intracellular survival within epithelial cells. These results confirm the role of the NADase to maintain intracellular survival of internalized GAS bacteria. On the other hand, planktonic bacteria used the M3 protein and SLS to maintain intracellular survival. This indicates that biofilm and planktonic bacteria of the M3 strain, once inside cells, behave differently and use different trafficking mechanisms hence the difference in localization and virulence factors needed to maintain prolonged persistence.

The precise role of M1 or M5 protein during biofilm formation is not clear since both were down-regulated in biofilm bacteria. However, presence or absence of these proteins did not affect the association, uptake, or persistence, of their corresponding strains thus indicating presence of other proteins in the M1 or M5 strains needed to mediate these processes.

Overall, these results indicate that the role of virulence factors during biofilm formation or intracellular persistence is not homogenous *in vitro*. Therefore, GAS seems to differentially regulate the expression of proteins during different stages of pathogenesis such as colonization and biofilm formation, or uptake and persistence. However, it should be taken into consideration that our model has limitations in which it does not fully mimic the respiratory environment and therefore biofilm bacteria might behave differently and have different expression profiles *in vivo*. To confirm our observed findings, *in vivo* models colonized with biofilm bacteria expressing or lacking these proteins could help us determine the role of virulence factors in GAS pathogenesis.

If similar findings are obtained *in vivo*, then therapeutics targeting proteins involved in biofilm formation should be different from those targeting uptake and persistence proteins. Additionally, treatment of recurrent respiratory infections mediated by

GAS should include high doses of intracellular antibiotics that are able to enter epithelial cells and kill GAS biofilm bacteria residing intracellularly.

Chapter 6: General summary and clinical significance of this thesis

“The aim of medicine is to prevent disease and prolong life, the ideal of medicine is to eliminate the need of a physician” – William James Mayo (1928)

To prevent human respiratory infections mediated by GAS, one should first understand how these bacteria function, and become familiar with the lifestyle and mechanisms these bacteria use to escape killing within the host. In this thesis, we introduced a possible treatment alternative to combat streptococcal infections mediated by antibiotic resistant isolates and investigated the bacterial lifestyle of GAS during biofilm formation and cellular uptake into epithelial cells *in vitro* via models mimicking, to some extent, the respiratory environment within the human body.

Important highlights

Multi-drug resistant streptococci (Spn, GAS, or GBS) have become a threat to global health by which limited treatment options to cure infections are present [3]. In this thesis, we introduced HAMLET as a potential treatment alternative for respiratory infections caused by these species. HAMLET is a bacteriostatic and bactericidal compound targeting drug resistant streptococci growing planktonically, regardless of their serotype or antibiotic resistance mechanism. By means of sodium and calcium ion transport, HAMLET mediated bacterial membrane depolarization and permeabilization which consequently lead to membrane rupture and death of these species. Using same mechanisms in all tested streptococci, HAMLET potentiated the erythromycin activity and reduced the erythromycin MIC down to sensitivity levels in isolates resistant to this antibiotic, regardless of the expressed erythromycin resistance mechanism or serotype. Additionally, the observed activity of HAMLET and erythromycin combination treatment was more potent than that of penicillin and erythromycin combined. Therefore, HAMLET alone or in combination with antibiotics is a potential therapeutic against respiratory infections caused by streptococci.

The impermeable biofilm structure and dormant state of biofilm bacteria is a form of antibiotic avoidance mechanism to escape the host- or cell-mediated killing. Biofilm formation in GAS has been documented, however bacteria were grown on surfaces that don't well represent its physiological niche within the respiratory tract. In this thesis, we optimized the pneumococcal biofilm model and used it as a tool to study colonization and biofilm formation of GAS on pre-fixed epithelial surfaces that, as compared to abiotic surfaces, better represent the physiological niche of GAS during respiratory infections. We showed that auto-aggregation and adhesion to epithelial cells are not major determinants of biofilm formation in GAS. Using biofilm bacteria grown in this model, protein and gene expression profiles of GAS biofilms were analyzed. We showed that biofilm formation is common in GAS and, depending on the strain, biofilm phenotypes and proteomes range between dense and functional biofilms of down-regulated protein expression (in M5 and M18) to thin and less-functional biofilms with up-regulated protein expression (in MIT1 and M3). Biofilm formation in GAS strains commonly involves 6 down-regulated proteins involved in the amino acid metabolism of arginine and other processes, and a network of 75 up-regulated proteins involved in transport, DNA/RNA metabolism, carbohydrate metabolism, translation, and transcriptional regulation of other proteins such as the CovR response regulator (part of the CovRS regulatory system).

The ability of GAS to form dense and well-developed biofilms was associated with up-regulated protein expression of proteins involved in carbohydrate metabolism, cell wall biogenesis, translation and transport, along with up-regulated expression (gene and protein expression) of the CovRS regulator. The CovRS system in turn represses the gene and protein expression of the biofilm inhibitory proteins, capsule biosynthesis protein HasA and cysteine protease SpeB. In the M3 strain, a network of bacterial chains coated with ECM containing DNA and proteins formed thin biofilms that required the presence of NADase and absence of SpeB and SLS. Biofilms of this isolate (as well as MIT1) failed to suppress CovRS gene expression and therefore capsule production was up-regulated which in turn resulted in the observed biofilm phenotype (thin and less developed biofilms).

Intracellular persistence of GAS bacteria is another form of antibiotic avoidance mechanism that help intracellular bacteria escape the antibiotic- or host-mediated killing. Here, the live cell infection model was presented as an *in vitro* tool to investigate uptake of GAS into respiratory epithelial cells. It was used to determine the association, internalization, persistence, and intracellular localization of biofilm bacteria. Confirming previous studies, we showed that biofilm bacteria of GAS isolates, regardless of their biofilm forming ability and proteome profiles, internalized and persisted equally (more than 44 h) within respiratory epithelial cells. Using the M3 protein, biofilm bacteria of the M3 isolate were taken up as large aggregates, through cellular actin and dynamin, that co-localized with actin-filaments within the perinuclear region of the epithelial cells, in the absence of SLO.

The prolonged intracellular persistence of biofilm bacteria required the expression of the M3 protein, hyaluronic acid capsule, NADase and SpeB.

Therefore, in the M3 strain, proteins involved in biofilm formation are different from those involved in cellular uptake or persistence of biofilm bacteria, which suggest differential regulation of bacterial protein expression during each stage in GAS pathogenesis. Moreover, to eliminate intracellular biofilm bacteria, higher doses of intracellular antibiotics should be used to treat respiratory infections caused by GAS.

Future plans

If time allowed, more investigations would have been done to get a clear picture of GAS mediated colonization and pathogenesis during respiratory infections and find better treatment approaches. There are still missing pieces of information in this puzzle that need to be addressed, below are few examples of what needs to be done:

In HAMLET

- The mechanisms behind HAMLET potentiated antibiotic activity in streptococci needs to be further addressed, for instance tracking the uptake of fluorescent antibiotics can be used to determine if this activity is due to high antibiotic doses trapped into the bacterial interior thereby killing the bacteria.
- As we introduced biofilm formation and cellular internalization by biofilm bacteria in GAS, it could be of interest to test if HAMLET alone or in combination with antibiotics could kill biofilm bacteria present extracellularly onto or intracellularly within epithelial cells using *in vitro* or *in vivo* settings.
- Complying with the guidelines to make safe drugs [171], combination treatment using recombinant HAMLET and antibiotics mediated the same potentiated activity in Spn (unpublished results) and *M. tuberculosis* as HAMLET purified from human milk [117]. It would be therefore of interest to see if recombinant HAMLET also potentiates the antibiotic activity in streptococci (Spn, GAS, or GBS) using *in vitro* and *in vivo* settings.

In GAS colonization and biofilm formation

- As the structural integrity of GAS biofilms formed on pre-fixed respiratory epithelial cells was disrupted by enzymes targeting ECM components (such as DNA or proteins), it would be of interest to see if the ECM of biofilms formed on pre-fixed keratinocytes also contain these components and whether enzymatic treatment will disrupt the structural integrity of these biofilms.
- As CovRS is commonly up-regulated in GAS biofilms, mutants lacking this regulator should be acquired and tested for its role during biofilm formation in GAS.
- As transmission of infection is thought to be mediated via biofilm bacteria instead of planktonic bacteria, live epithelial cells seeded with biofilm bacteria should be investigated for biofilm formation over time. In live keratinocytes, biofilm bacteria were less toxic than planktonic bacteria and survived for longer periods of time on the surface of these cells [29], but whether biofilm formation on live respiratory epithelial cells show similar results is not known and needs to be further investigated.

In uptake and persistence of GAS bacteria

- Determine the regulation of other proteins involved during GAS uptake and persistence, where proteomic profiles of intracellular bacteria isolated from cell lysates at different time points after infection can be analyzed and compared to that of seeded biofilm bacteria.
- Live imaging to track bacterial uptake into epithelial cells over time is needed to determine the involved trafficking pathways and bacterial localization within cells.
- To address oropharyngeal infections *in vitro*, biofilm bacteria formed on keratinocytes (pre-fixed or live) should be tested for their ability to internalize and persist within live keratinocytes. The cellular uptake pathways and virulence mechanisms involved can then be determined.
- As mice lack tonsils, GAS colonization of the functionally equivalent nasal associated lymphoid tissue (NALT) in mice has been earlier used as an *in vivo* model mimicking human oropharyngeal infections [29, 172, 173]. The CFU/ml of intracellular bacteria obtained from isolated NALT over time can be used to study the internalization and persistence levels *in vivo*. The proteomic profiles of isolated bacteria can be used to determine proteins involved in pharyngeal infections *in vivo*. GAS strains lacking or expressing the CovRS regulon or other virulence factors can be used to determine the involved virulence mechanisms.

Clinical significance

Results presented in this thesis suggest a potential therapeutic role of HAMLET in repurposing antibiotics currently causing treatment failures in patients suffering from streptococcal infections. Additionally, this thesis could have clinical implications and help in better understanding the colonization and biofilm formation process, the role of biofilms during GAS pathogenesis, and the mechanisms used by these bacteria to escape antibiotic treatment. This in turn has the potential to aid in improving treatments of GAS infections, such as strep throat, otitis media, and pneumonia. Identifying virulence mechanisms involved in biofilm formation and invasion will help in identifying potential therapeutic targets that could interfere with GAS pathogenesis.

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Antibiotic resistance and pathogenesis of Streptococci with focus on Group A Streptococci

It's 2020, we are in the middle of a pandemic outbreak of the corona virus (Covid-19) and the most common instructions we receive today, to prevent the spread, include hand washing, sanitizing, and staying in quarantine when harboring respiratory symptoms. But where did these instructions originate from and how are they known to limit the spread of the microbe?

If we go back in time, particularly the 7th century BC, infected people were asked to isolate themselves until symptoms disappeared. It was not until the 14th century, during the black death plague pandemic, where the term "quarantine" was used for the first time. In the 17th century, certain parts of the world suffered from an epidemic outbreak of respiratory infections (scarlet fever) caused by the human pathogen *Streptococcus pyogenes*, by which patients expressing respiratory symptoms were asked to stay in quarantine. Later, another outbreak affecting pregnant women and newborns during childbirth (puerperal fever) mediated by *Streptococcus agalactiae*, started. Spread of the infection was common in pregnant women who had been in contact with healthcare workers. Lack of sanitation and hand washing procedures among healthcare workers, were identified as the cause of the spread and since then the importance of these procedures in preventing microbial spread, was recognized. In the 20th century, a deadly Spanish flu pandemic started. Patients suffering from severe respiratory infections often had a co-infection of the influenza virus along with *Streptococcus pneumoniae*, that was in most cases fatal.

These streptococcal types commonly form a global threat to human health due to the increased spread of antibiotic resistant infections. Treatment choices are limited, and new treatment alternatives are therefore needed. Accordingly, the aim of this thesis is to provide potential therapeutic alternatives such as the human milk complex HAMLET that targets and reduces antibiotic resistance in these species. Additionally, the mechanisms and factors used by *Streptococcus pyogenes* during disease development are investigated here, which will help in identifying potential therapeutic targets that could interfere with infections caused by this pathogen.