Örebro Studies in Medicine 333

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Antimicrobial peptides for topical treatment of bacterial wound infections

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Title: Antimicrobial peptides for topical treatment of bacterial wound infections

Publisher: Örebro University, 2025
www.oru.se/publikationer

Print: Örebro University, Repro 08/2025
Cover image by Hannele Åhlén

ISSN: 1652-4063
ISBN: 978-91-7529-684-5 (print)
ISBN: 978-91-7529-685-2 (pdf)

Abstract

Modern medicine relies on the access to effective antibiotics. They are not only necessary to treat infections but enable the invasive therapies and surgeries to which we are accustomed today. Hence, the significant rise of bacterial resistance towards antibiotics threatens to topple a large part of global health care. This thesis investigates the potential of two antimicrobial peptides (AMPs), namely the bacteriocin Plantaricin NC8 αβ (PLNC8 αβ), and a novel synthetic lipopeptide derived from PLNC8 β termed 6-C5-N, for the topical treatment of infected wounds. Through a series of studies, the effectiveness and broad-spectrum activity of both these AMPs *in vitro* is demonstrated, and their influence on human cells in regard to toxicity, inflammation and survival is evaluated. Both AMPs exhibit low cytotoxicity *in vitro* and modulate important cytokines and growth factors in relation to infection and wound healing. Furthermore, utilizing *ex vivo* and *in vivo* models, it is demonstrated that 6-C5-N is an interesting candidate for the topical treatment of infected wounds. Additionally, a possible explanation of the complex problem with bacterial resistance to AMPs is presented, by demonstrating how extracellular divalent cations can be utilized by gram negative bacteria as protection against positively charged antibacterial peptides. In conclusion, PLNC8 αβ and its derivative lipopeptide 6-C5-N are promising candidates for topical treatment of infected tissues and could play a role in the struggle against the development of antimicrobial resistance.

Keywords: Antibiotic resistance, antimicrobial peptides, bacteriocin, plantaricin, lipopeptides, ESKAPE, pathogens, chronic wounds, wound healing

For Bo and Linnéa

As you set out for Ithaka
hope your road is a long one,
full of adventure, full of discovery.
Laistrygonians, Cyclops,
angry Poseidon—don't be afraid of them:
you'll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement
stirs your spirit and your body.
Laistrygonians, Cyclops,
wild Poseidon—you won't encounter them
unless you bring them along inside your soul,
unless your soul sets them up in front of you.

Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.

Ithaka gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithakas mean.

C. P. CAVAFY

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List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Musa A, **Wiman E**, Selegård R, Aili D, Bengtsson T, Khalaf H. *Plantaricin NC8 αβ prevents Staphylococcus aureus-mediated cytotoxicity and inflammatory responses of human keratinocytes*. Sci Rep. 2021 Jun 15;11(1):12514. doi: 10.1038/s41598-021-91682-6.

II. **Wiman E**, Zattarin E, Aili D, Bengtsson T, Selegård R, Khalaf H. *Development of novel broad-spectrum antimicrobial lipopeptides derived from plantaricin NC8 β*. Sci Rep. 2023 Mar 13;13(1):4104. doi: 10.1038/s41598-023-31185-8.

III. **Wiman E**, Sotra Z, Rinklake I, Häll J, Junker JPE, Aili D, Bengtsson T, Khalaf H. *The antibacterial lipopeptide 6-C5-N effectively combats Staphylococcus aureus, counteracts bacterial cytotoxicity and promotes wound healing.* (Submitted).

IV. **Wiman E**, Palm K, Nyström G, Junker JPE, Aili D, Bengtsson T, Khalaf H. *Gram negative bacteria utilize extracellular divalent cations as a defence mechanism against positively charged antimicrobial peptides.* (Manuscript).

Other works not included in thesis

I. Eskilson O, Wiman E, Reustle N, Langwagen J, Sotra Z, Svärd A, Selegård R, Baş Y, Berglund L, Oksman K, Bengtsson T, Junker JPE, Khalaf H, Aili D. *Nanocellulose Wound Dressings with Integrated Protease Sensors for Detection of Wound Pathogens.* ACS Sens. 2025 May 20. doi: 10.1021/acssensors.4c03428.

II. Zattarin E, Sotra Z, Wiman E, Bas Y, Rakar J, Berglund L, Starkenberg A, Björk EM, Khalaf H, Oksman K, Bengtsson T, Junker JPE, Aili D. *Controlled release of antimicrobial peptides from nanocellulose wound dressings for treatment of wound infections*. Mater Today Bio. 2025 Apr 10;32:101756. doi: 10.1016/j.mtbio.2025.101756.

III. Eskilson O, Zattarin E, Berglund L, Oksman K, Hanna K, Rakar J, Sivlér P, Skog M, Rinklake I, Shamasha R, Sotra Z, Starkenberg A, Odén M, Wiman E, Khalaf H, Bengtsson T, Junker JPE, Selegård R, Björk EM, Aili D. *Nanocellulose composite wound dressings for real-time pH wound monitoring.* Mater Today Bio. 2023 Feb 6;19:100574. doi: 10.1016/j.mtbio.2023.100574.

IV. Bengtsson T, Zhang B, Selegård R, Wiman E, Aili D, Khalaf H. *Dual action of bacteriocin PLNC8 αβ through inhibition of Porphyromonas gingivalis infection and promotion of cell proliferation.* Pathog Dis. 2017 Jul 31;75(5):ftx064. doi: 10.1093/femspd/ftx064

List of abbreviations

AB – Antibiotics
AMP – Antimicrobial peptides
AMR – Antimicrobial resistance
APC – Antigen-presenting cell
CAC – Critical aggregation concentration
CD – Circular dichroism
CF – Carboxyfluorescin
CXCL-8 – C-X-C motif chemokine ligand 8
DAP – Diaminopimelic acid
DIC – Diisopropylcarbodiimide
DIEA – Dipropylethylamine
DLS – Dynamic light scattering
DMF – Dimethylformamide
EAEC – Enteroaggregative *E. coli*
ECC – Enterobacter cloacae complex
EHEC – Enterohemorrhagic *E. coli*
ELISA – Enzyme-linked immunosorbent assay
EPS – Extracellular polymeric substance
ESBL – Extended spectrum β-lactamase
ET – Exfoliative toxins
FBC – Fractional bactericidal concentration
FBS – Fetal bovine serum
FIC – Fractional inhibitory concentration
GF – Growth factor
GFP – Green fluorescent protein
IL – Interleukin
Kdo – 3-deoxy-d-manno-octulosonic acid
LDH – Lactate dehydrogenase
LPS – Lipopolysaccharide
LTA – Lipoteichoic acid
MAPK – Mitogen-activated protein kinase
MBC – Minimal bactericidal concentration
MIC – Minimal inhibitory concentration
MMP – Matrix metalloprotease
MOI – Multiplicity of infection

MRSA – Methicillin-resistant *S. aureus*
MSSA – Methicillin-sensitive *S. aureus*
NAG – *N*-acetylglucosamine
NAM – *N*-acetylmuramic acid
NFκB – Nuclear factor-κB
NOD – Nuclear oligomerization domain
OMV – Outer membrane vesicle
PAMP – Pathogen-associated molecular pattern
PBP – Penicillin-binding protein
PBS – Phosphate-buffered saline
POPC – 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPG – 1-Hexadecanoyl-2-(9Z-Octadecenoyl)-sn-Glycero-3-Phospho-glycerol
POPS – 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine
PRR – Pattern-recognition receptor
SAg – Superantigen
SE – Staphylococcal enterotoxin
TLR – Toll-like receptor
TFA – Trifluoroacetic acid
UPEC – Uropathogenic *E. coli*
VRE – Vancomycin-resistant *Enterococcus spp.*
WTA – Wall teichoic acid

Introduction

The world of bacteria

Bacteria are found in abundance in all the explored parts of the world, from the most extreme environments such as volcanoes, hot springs reaching temperatures above the boiling point, and the deepest trenches of the sea; to the more mundane milieus such as soil, water, and the human body [1-3]. While at glance nothing more than a simple unicellular organism, bacteria are nevertheless complex organisms; proven survivors that are quick to adapt and evolve. Indeed, should one ever find oneself in need of proof on the theory of evolution, one can just mention the speed with which bacteria exposed to sub-lethal doses of an antibiotic can develop resistance and become immune [4-6]. (The topic of bacterial resistance to antibacterial agents is as interesting as it is important and will be discussed in a separate section below.) Bacteria are generally found in sizes between 0.1 and 1 µm and thus require the aid of a microscope to be visible to humans [7]. (*Thiomargarita namibiensis*, the world's second largest known bacterium, can reach sizes up to 0.75 mm and is visible to the naked eye [8]). The procaryotic bacteria are divided into the *archaebacteria* and the *eubacteria*, or so-called *true bacteria* [9]. In this thesis, the focus is solely on the *eubacteria*, and all further references to bacteria henceforth is thus considered to be referencing *eubacteria*.

Classification and structure

Universal to all bacteria is the lack of a nucleus, 70S ribosomes, a smaller (compared to eukaryotes) and predominantly circular genome, lack of other specialized organelles and cellular respiration taking place in the plasma membrane, akin to our mitochondria [10-12]. In fact, the endosymbiotic theory postulates that mitochondria and chloroplasts were once procaryotic organisms, and that eucaryotic cells evolved through a symbiotic relationship between procaryotic cells [13]. Furthermore, bacteria can be aerobic, facultative anaerobic or strictly anaerobic. The classification of bacteria is based on their macroscopic and microscopic morphology, the metabolic and growth characteristics, genotype, and the structure and composition of their

cell wall and phospholipid bilayers [14]. It is beyond the scope of this thesis to provide detailed descriptions of the many intricacies pertaining bacterial classification; however, a deeper understanding of the bacterial cell wall and cell membrane structure is necessary, and a more thorough description is thus warranted.

The bacterial cell wall is composed of peptidoglycan, also known as murein. Peptidoglycan surrounds the cytoplasmic membrane and provides a rigid layer conferring stability to the cell and is found in all eubacteria with the exception of *Chlamydia spp*. and *Mycoplasma spp*., who instead use host-derived sterols to regulate their membrane fluidity [15-17]. The structure of peptidoglycan is rather elegant in its simplicity, consisting of strands of repeating disaccharides of alternating *N*-acetylglucosamine (Glc*N*Ac, NAG) and *N*-acetylmuramic acid (Mur*N*Ac, NAM) residues, linked by β-1→4 bonds. Extending from each Mur*N*Ac residue is a small stem oligopeptide, usually consisting of four amino acid residues, which normally are *l*-Alanine (*l*-Ala), *d*-Glutamic acid (*d*-Glu), *meso*-DAP (2,6-diaminopimelic acid, DAP, a precursor to *l*-Lysine) and finally two *d*-Alanines (*d*-Ala). These oligopeptides are in turn cross-linked to another Mur*N*Ac residue by a polypeptide bridge, thus linking the polysaccharide strands by peptide bonds [18].

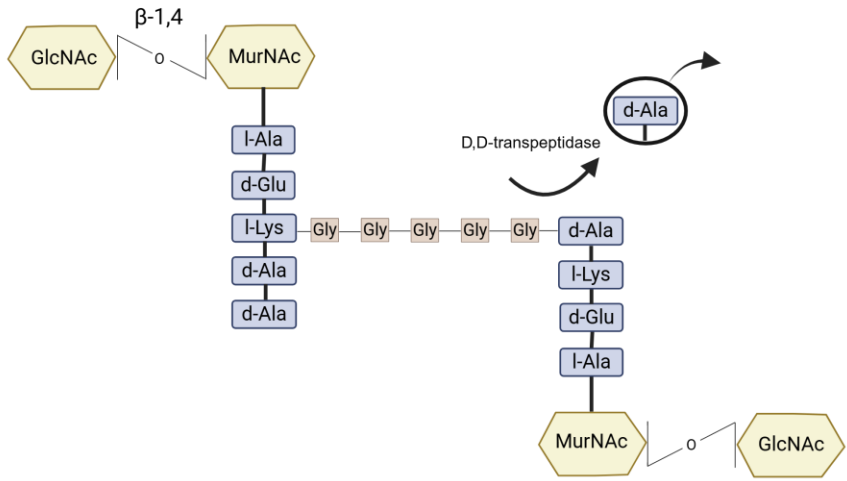

Figure 1. Peptidoglycan structure of *Staphylococcus aureus*, showing the final cross-linking reaction between two Mur*N*Ac sugars facilitated by *D,D*-transpeptidase (a penicillin-binding protein), connecting the pentaglycine bridge with the fourth *d*-Ala of the nascent Mur*N*Ac, simultaneously releasing one *d*-Ala residue. (Adapted from Arbeloa et al. [16])

While the details presented in figure 1 are specific for *Staphylococcus aureus*, the features described above are found in all known bacterial species, albeit with minor variations in the positions of the bonds or composition of the oligopeptide stem or polypeptide bridge [19]. Not only does peptidoglycan provide rigorous strength and stability to the bacterium, but it also allows for the passage of relatively large molecules, including proteins, making peptidoglycan an essential functional part of the bacterial cell wall [20]. During synthesis of peptidoglycan, a precursor molecule, lipid II, is synthesized in the cytoplasm, close to the lipid bilayer. Lipid II is then transported, or "flipped", across the inner leaflet by integral membrane proteins [21]. The specifics of these integral membrane proteins are still being explored, but the SEDS (shape, elongation, division and sporulation) protein FtsW and the MurJ protein (belonging to the MOP [multidrug, oligosaccharide-lipid, polysaccharide] superfamily of

transporter proteins) have been identified as possible lipid II-flippases [22, 23]. Lipid II is then used as substrate for the glycosyltransferases that polymerize the peptidoglycan strands, after which the strands are linked to nascent strands by transpeptidases facilitating the peptide bonds [21]. Importantly, both these reaction steps are catalysed by the enzymatic activity of transpeptidases belonging to the class A penicillin-binding proteins (aPBPs) [24]. As implied by the name, the PBPs are important targets for the β-lactam group of antibiotics (which includes penicillin) and will be discussed further in the antimicrobial resistance section.

Peptidoglycan is universal among bacterial species; however, its' thickness is not. Furthermore, all bacteria have a lipid bilayer membrane on the inside of the peptidoglycan (or sacculus), but some species also possess an outer membrane, enveloping the peptidoglycan. This is the basis for the now established division of bacteria into gram positive or gram negative. In the late 19th century, the Danish physician Hans Christian Gram, at the time working in the pathology department at the municipal hospital in Berlin, was working on developing a staining method for obtaining blue nuclei in kidney sections, while simultaneously staining the cytoplasm brown. In a serendipitous turn of accidental events, he noticed not only that cells stained with aniline-gentian violet and then counterstained with iodine-potassium iodide were completely decolorized when soaked in alcohol, but also that some bacterial species were resistant to this decolorization [25]. Gram himself did not fully understand the impact of his discovery, and for a long time it was primarily used to distinguish bacteria from tissue in histological slides [26]. We now know however, that the intransigence to decolorization in gram positive bacteria is due to a thick layer of peptidoglycan, which, when exposed to alcohol, dehydrates and shrinks, which decrease permeability and thus retains the stain. In contrast, gram negative bacteria have a very thin layer of peptidoglycan, usually consisting of only a mono- or bi-layer, not thick enough to contain the stain. Moreover, gram negative bacteria possess an outer lipid membrane, that is susceptible to being dissolved by alcohol, thereby allowing the stain to escape [27]. Hence, the cell wall of a gram positive bacterium consists of an inner membrane, surrounded by a thick layer of peptidoglycan, whereas the gram negative

bacterium possesses both an inner and an outer membrane, with a thin mono- or bi-layer of peptidoglycan residing between these two membranes [28].

Within in the cell wall of gram positive bacteria are found teichoic acids (TAs), constituting over half of the total mass of the cell wall. Primarily, they maintain cell morphology and confer rigidity but are also important for ion exchange and control of enzymatic activity important for cell wall turnover [29]. TAs are linear, anionic glycopolymers of ribitol- or glycerol-phosphates linked by phosphodiester bonds, and are either anchored to peptidoglycan (wall teichoic acids, WTA) or to the bacterial membrane as lipoteichoic acid (LTA) [30, 31]. The majority of TAs have zwitterionic properties, and are highly diverse, even intra-species [32]. Considered as bacterial virulence factors, TAs also aid the bacterium in preventing passage of potentially harmful molecules through the peptidoglycan layer, facilitate adherence to host cells, tissues, and bacterial biofilm [33]. Furthermore, they are activators of the human innate immune system, acting as antigens [31], which will be further discussed in subsequent sections.

As mentioned, gram negative bacteria possess not only an inner membrane, but also an outer membrane. The outer membrane of the gram negative bacteria, however, is not quite like other biological membranes. While it is a lipid bilayer, only the inner leaflet contains phospholipids. The outer leaflet is instead composed of glycolipids, which, with a few exceptions, are constituted by the important molecule lipopolysaccharide (LPS) [34]. LPS is an intricate and important structure and is further described below. Between the two lipid bilayers in gram negative bacteria is the periplasmic space. Here, many important cellular mechanisms take place, such as protein maturation, regulation of signalling functions and the sequestration of toxic molecules [35]. In conclusion, the cell walls of both gram positive and gram negative bacteria are complex structures essential for bacterial viability and confers a net negative charge to the bacterium.

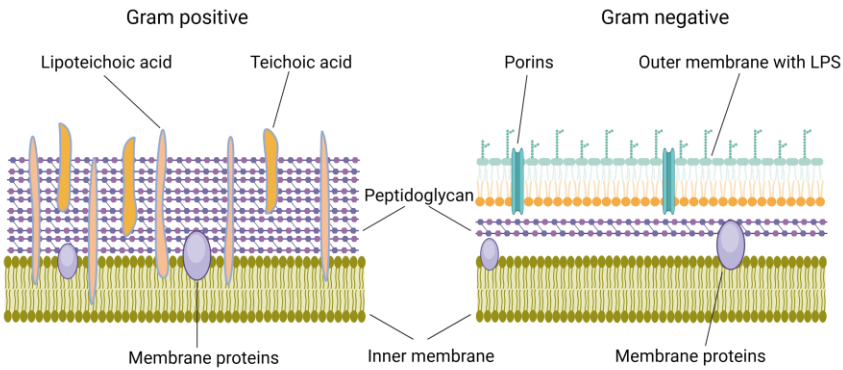

Figure 2. The gram positive (left) and gram negative (right) cell wall.

Lipopolysaccharide

The negatively charged, polyanionic lipid LPS is found on the outer leaflet of the outer lipid bilayer of gram negative bacteria. LPS consists of three major sections. Innermost, it is made up of Lipid A, then extending outward in the core oligosaccharide followed by the O-antigen, both of which are linked to lipid A via 3-deoxy-d-manno-octulosonic acid (Kdo) [36-38].

During synthesis of LPS, the Kdo_2-lipid A part (considered an essential structure in the majority of gram negative bacteria, required for bacterial growth and viability [39]) of LPS is synthesised in the cytoplasm, catalysed by nine different evolutionally conserved enzymes. The Kdo_2-lipid A is then transported to the outer leaflet via flippases, where it acts as an anchor, bridging the core oligosaccharide and O-antigen with the inner leaflet. After synthesis, LPS generally further undergoes several modifications via molecules that are not as genetically conserved, making up for part of the variations betwixt different gram negative species [36, 40].

The core oligosaccharide is made up of Kdo, heptoses and hexoses, and can be further divided into two parts, an inner and an outer core. The number of sugars in the core oligosaccharide varies between species, but typically 4-5 sugars are present [34]. As is the case with LPS, the majority of research is done on *E. coli*, where the genes regulating

oligosaccharide synthesis can be found on the *waa* locus. After synthesis, the core oligosaccharide is attached to Kdo_2-lipid A before it is transferred (flipped) to the surface [41]. Importantly, negatively charged phosphate residues in the heptose molecules found in the core oligosaccharide allow for the crosslinking of adjacent LPS via divalent cations [42]. Attached to the core oligosaccharide is the O-antigen, encompassing the surface of the cell and extending into the environment [43]. The hydrophilic O-antigen chains are made up of repeating subunits of a variety of different sugars, some of which are rarely found elsewhere [44]. The number of repeating polysaccharides also varies within a wide range, up to 40 residues, which combined with the equally large variety of sugar residue types lends itself to a substantial variance among different O-antigen chains [45].

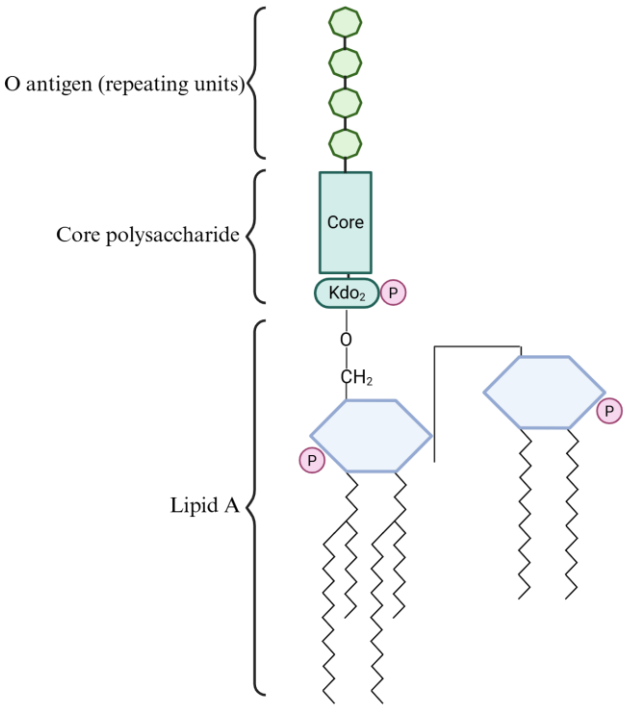

Figure 3. Lipopolysaccharide. The three major components of LPS are shown; lipid A with the disaccharide phosphates, core polysaccharide with Kdo, and the O-antigen which is made up of up to 40 repeating units and may or may not be present depending on the species or strain.

LPS is also known as endotoxin, as it acts as a potent stimulator of the human innate immune system [46]. Lipid A is considered to be the structure responsible for the toxic effects of LPS [47], and this host-pathogen interaction will be further discussed in coming sections. As previously discussed, most of the research on LPS have been performed in the gram negative bacterium *Escherichia coli*, especially the widely used laboratory K-12 strain [48], and the structure of LPS is not uniform among bacterial species or strains. The K-12 strain does, for instance, not contain the O-antigen, and its LPS is thus referred to as "rough", whereas wild-type *E. coli* possessing the O-antigen has "smooth" LPS [28]. While the fundamental structure and biosynthesis is generally the same, the heterogenicity of LPS in different species and strains should be noted, as it can affect the virulence of the bacterium and hence be of clinical importance [49].

Outer membrane vesicles

Closely related to the function of gram negative bacteria and their envelope, are the outer membrane vesicles (OMVs), produced by almost all known gram negative bacteria. These are small, globular vesicles of around 20-200 nm originating from budding parts of the outer membrane of the gram negative bacterium. They are pinched off, and released into the surrounding environment, where they facilitate numerous functions such as quorum sensing (see biofilm section below), acquisition of nutrients and transfer of genetic material [50]. Importantly, being derived from the outer membrane, they are encompassed by variations of LPS [51], and as mentioned, LPS, or endotoxin, is highly immunogenic. Furthermore, several bacterial toxins and other immunomodulating molecules are released through OMVs, and thus OMVs play a major role in gram negative bacterial pathogenesis [52]. OMVs can also constitute a mechanism of evasion of antibacterial agents, by releasing proteases or absorbing the harmful molecules preventing them from exercising their antibacterial effects [53, 54]. Additionally, by nature of their origin, OMVs possess the negative charge intrinsic to the outer membrane and could thus act as decoy targets for positively charged antibacterial drugs relying on electrostatic interactions for their activity [55].

Biofilm

Even though they are unicellular organisms, several bacteria have the ability to adopt a form of multicellular society by forming biofilms. These are defined as bacterial communities attached to a surface, which can be of artificial or natural origin, where they surround themselves with a slimy film of extracellular polymer matrix [56]. The bacteria produce this matrix themselves, however some host derived components such as fibrin and immunoglobins are often present as well [57]. Bacterial biofilms are commonly found in nature, where they have been a contributing factor to the exceptional survival skill of bacteria. Consequently, when pathogenic bacteria form biofilm on surfaces present in or on humans, they can become very problematic [58]. In fact, whether related to implant infection (e.g. catheters and prosthetic joints) or tissue infections (e.g. otitis media, endocarditis and chronic wound infections), bacterial infections are predominantly mediated by biofilm formation [57, 59]. Mature biofilms are often polymicrobial, consisting of different species of bacteria and sometimes including fungi and even viruses [60, 61]. This polymicrobial nature combined with the severalfold increase in antimicrobial resistance conferred by the defensive properties of biofilm make these infections very difficult to treat [62].

The initiation of biofilm formation usually occurs as a response to environmental stress, beginning with adhesion followed by subsequent reprogramming of bacterial gene expression and metabolism arming the bacteria with a multitude of ways to combat the external challenges [63, 64]. The initial reversible adhesion of planktonic bacteria to a surface is generally weak and primarily mediated by electrostatic or van der Waals forces and hydrophilic interactions [65]. As more cells accumulate, they utilize bacterial adhesion molecules such as fimbria and flagella, in combination with covalent bonding between cell surface structures, to form stronger, irreversible attachments [65, 66]. At this stage, the bacteria communicate via a type of chemical signalling known as quorum sensing, which modulates the above mentioned cellular reprogramming within the bacterial community [67, 68]. The following step in biofilm formation is the synthesis of the polysaccharides, proteins, nucleic acids and lipids that collectively

make up the extracellular polymeric substances (EPS) [69, 70]. Notable EPS are the Curli amyloids found within *E. coli* biofilms [71, 72], and Pel and Psl in *P. aeruginosa* biofilms [73-75]. Together with a considerable amount of water, this bacterial extracellular matrix (ECM) further promotes adhesion and condensation [76]. During maturation, the size of the ECM increase, and water channels are formed, creating a network for the transfer of nutrients and signalling molecules [56, 63, 77, 78]. Cells residing in the biofilm adopts different metabolic states depending on their localization within the ECM affecting their ability to acquire nutrients. A small portion of the bacterial population within a biofilm may enter an almost dormant state, in which they are highly resistant to environmental stress. Known as persister cells, they can survive for long periods of time in unfavourable conditions. However, if the conditions change to a more favourable state, they can become metabolically active again and start to proliferate [79]. Furthermore, some bacteria can return to their planktonic state and leave the biofilm, a process known as dispersion, further disseminating the infection [80, 81]. In conclusion, bacterial biofilm is a complex structure, capable on conferring significant advantages to the bacteria residing within. Moreover, not only does biofilm formation provide a physical barrier against the immune system and exogenous antibiotics, but the cellular remodelling and communication between the bacteria in biofilm allows for transfer of information providing additional methods for antimicrobial resistance [82-86]. Further details pertaining to antimicrobial resistance mechanisms are reviewed in a separate section.

Pathogenic bacteria

Not all bacteria are pathogenic to humans. In fact, many bacterial species are involved in symbiotic or commensal relationships with humans where either both species benefit, or where one species (usually the bacterium) benefit and the other is indifferent, neither hurting nor benefiting [87-89]. For example, human gut microbiota including the so called probiotic bacteria residing in our gastrointestinal tract confer several health benefits to human, such as facilitating metabolism of ingested nutrients, providing defence against pathogens and being responsible for the synthesis of important vitamins [90-92].

There are, however, several pathogenic species present in our surroundings or even on or within our bodies. These can be either opportunistic pathogens, utilizing a temporary loss of physical barrier function or immunosuppression to invade the host and cause disease, or primary pathogens, who are capable of causing disease irrespective to the functional and immunological condition of the host [93]. Central to a bacterium's ability to cause disease is its presence of virulence factors. Thus a measure of a bacterium's pathogenicity, bacterial virulence encompasses several mechanisms facilitating invasion and adherence, immune system avoidance and finally tissue destruction. However, it should be noted that individual virulence factors rarely facilitate only one such mechanism but rather play roles in all [94-96]. The works in this thesis have primarily focused on the so called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.*) and *Escherichia coli*, described below, with special focus on *S. aureus* and *E. coli*. The ESKAPE pathogens are critical pathogenic bacteria often harbouring multidrug resistance profiles, recognized as one of the major threats to global health in the present and foreseeable future [97]. Resistant variants of the above mentioned pathogens are all found on the WHO Bacterial Priority Pathogens List, updated 2024 [98].

Enterococcus faecium is a gram positive opportunistic pathogen, commonly found in the gastrointestinal microbiota of mammals, that can cause severe infections and is often responsible for high morbidity in hospitalized patients [99]. *E. faecium* is exceptionally well adapted to survive on inorganic surfaces such has hospital beds and railings, and comes equipped with a multitude of virulence factors [100]. These include the aptly named cytolytic toxin Cytolysin [101], several proteases that facilitate colonization and provides nutrients, and a vast array of surface adhesion proteins promoting adhesion and biofilm formation [102, 103]. Furthermore, the global prevalence of vancomycin-resistant strains of *E. faecium* (VRE) is increasing, likely through horizontal gene transfer of the *vanA* and *vanB* genes [99].

Staphylococcus aureus is a gram positive, aerobic coccus commonly found as a commensal colonizing the skin and nares of humans [104].

It is an opportunistic pathogen, and one of the main causative agents of respiratory, soft tissue, wound, prosthetic joint and surgical site infections [105, 106]. The pathogenicity of *S. aureus* is dependent on the many virulence factors and cytotoxic mechanisms intrinsic to the bacterium. Notable examples are the capsule present in some strains, preventing opsonization and phagocytosis and the immunoglobulin-binding protein A preventing antibody-mediated elimination [107]. Furthermore, *S. aureus* produces several potent toxins. Common in most strains are the cytolytic alpha, beta, gamma and delta toxins, whereas more virulent strains also produce staphylococcal enterotoxins (SEs), exfoliative toxins (ETs) and toxic shock syndrome toxin-1 (TSST-1) [108]. The exfoliative toxins are involved in the pathogenicity of serious skin lesions such as bullous impetigo and staphylococcal scalded-skin syndrome (SSSS), where the exfoliative toxins A and B (ETA and ETB) target the desmosome desmoglein 1, disrupting cell-cell adhesion [109, 110]. TSST-1 and the SEs belong in a group of toxins known as superantigens (SAgs) [108]. These potent toxins are capable of directly binding to the major histocompatibility complex II on antigen-presenting cells (APCs), circumventing the normally occurring internalization, degradation and presentation occurring in APCs [111]. SAgs can then stimulate a conserved part of the variable β chain of the T-cell receptor, resulting in unspecific activation of up to 30% of the total T-cells within the host, causing a massive systemic inflammatory cascade [112].

Moreover, the horizontal acquisition of the *mecA* gene coding for an alternative, β-lactam-insensitive, penicillin-binding protein (PBP2a), has resulted in the global spread of methicillin-resistant *S. aureus*, commonly abbreviated as MRSA [113]. MRSA is notorious as causative pathogens of serious nosocomial or community-acquired infections [114]. Even more troubling is the emergence of vancomycin-resistant *S. aureus* (VRSA), obtained by acquisition of the *vanA* gene cluster found in VRE as mentioned above, considering Vancomycin is one of the remaining treatment options for MRSA [115].

The facultative anaerobic, rod-shaped, gram negative *Klebsiella pneumoniae* is commonly found asymptomatically colonizing the gastrointestinal tract, but can, if given the opportunity, cause pneumonia, and

represents up to one fifth of all gram negative sepsis cases [116, 117]. Known as exceptionally prone to acquire resistance, *K. pneumoniae* clones with simultaneous production of extended spectrum β lactamases (ESBLs) and carbapenemases, while at the same time possessing genes coding for resistance to aminoglycosides and quinolones, can be virtually impossible to treat [118-120]. This combination of resistance profiles has bestowed upon such *K. pneumoniae* clones the dubious honour of reaching the top position on the above mentioned WHO bacterial priority list [98]. *K. pneumoniae* virulence factors include the presence of a capsule, iron-siderophore complexes to compete with host uptake of free Fe^{3+} ions, and LPS and other outer membrane proteins [121].

Another opportunistic pathogen, *Acinetobacter baumannii*, is a gram negative bacillus that was sensitive to most antibiotics as late as the 1970s but has since acquired multi-drug resistance [122]. Accompanied by the other ESKAPE pathogens, it is now a major cause for problematic nosocomial infections [123]. Likely dependent on the fact that the emergence of clinically significant clones of *A. baumannii* is more recent, studies on their pathogenicity and virulence factors are not as extensive. Nevertheless, *A. baumannii* are excellent producers of biofilm, and the outer membrane protein OmpA (or Omp38) has been shown to bind to mitochondria and cause dysfunction, ultimately followed by apoptosis of the parent cell, contributing to the pathogenicity of the bacterium [124, 125]. As a gram negative bacterium, it also contains LPS, further promoting pathogenicity.

First discovered by Carle Gessard, the gram negative facultative aerobe *Pseudomonas aeruginosa* got its name from the blue and green coloration on bandages from French soldiers in the late 1880s, now known to stem from pyocyanin and pyoverdine [126]. Interestingly, both pyocyanin and pyoverdine are also virulence factors of *P. aeruginosa*, acting as exotoxins [127]. Often found in chronic wounds and the lungs of patients suffering from cystic fibrosis [128, 129], *P. aeruginosa* readily produces biofilm, where quorum sensing signalling molecules have been shown to regulate the expression of several virulence factors [129]. These includes exotoxins, proteases, siderophores and secretion system, in addition to pili and flagella for motility,

adherence and biofilm formation [130]. *P. aeruginosa* has a large genome which harbours many intrinsic resistance factors [131], and multi-drug resistant clones are abundant, including ESBL- and carbapenemases-producing strains [127, 132].

The gram negative, facultative anaerobic genus of *Enterobacter* is bacillus-shaped, and encompasses 22 species. One of those 22 is the *Enterobacter cloacae* complex (ECC), encompassing an additional seven species with similar pheno- and genotypes. Out of the 22 *Enterobacter spp.*, primarily the ECC, *E. aerogenes* and *E. hormaechei* are of clinical importance, and as is the case with all the ESKAPE pathogens, strains featuring multidrug resistance are abundant [133]. Interestingly, most *Enterobacter spp.* are inherently resistant to several β-lactam antibiotics, facilitated by expression of an inducible β-lactamase [134]. Similarly to *A. baumannii*, research on the virulence factors of *Enterobacter spp.* is sparse, but LPS, enterotoxins, secretions systems and flagella promoting colonization are implicated in pathogenesis [133].

The gram negative bacillus *Escherichia coli* has been widely used as a model organism, and much is known regarding its structure and pathogenicity. Several volumes could be (and have been) written on the subject of *E. coli*; however, only a brief overview of the most relevant factors related to this thesis will be presented here. A highly diverse species, pathogenic *E. coli* are grouped in multiple so called *pathovars*, i.e. groupings of strains with similar pathogenic profiles. Well known examples include the uropathogenic *E. coli* (UPEC) and enterohemorrhagic *E. coli* (EHEC). These locations (the urinary and gastrointestinal tract, respectively), with the addition of some strains causing meningitis, are the main sites of *E. coli* infection [135]. The list of known *E. coli* virulence factors is vast and describing them all is beyond the scope of this thesis. Notable virulence factors. include several types of fimbriae for host adhesion including Curli amyloids promoting biofilm formation, several types of siderophores promoting survival in blood and urine, endotoxin and numerous other toxins [136, 137]. Important toxins are the Shiga toxins, responsible for the haemorrhagic diarrhoea EHEC [138]. Some might remember the 2011 outbreak in Germany of the novel EHEC strain O104:H4, causing significant morbidity and multiple deaths. Also found in

neighbouring countries in Europe, the spread of O104:H4 was believed to be caused by sprouts cultivated from contaminated Fenugreek seeds originating from Egypt. Interestingly, O104:H4 was found not only to possess virulence factors from EHEC strains (such as the Shiga toxins) but also harboured plasmids containing virulence factors from enteroaggregative *E. coli* (EAEC), resulting in a hypervirulent strain [139, 140]. To summarize, *E. coli* is a very important bacterium that has helped us generate vast amounts of knowledge in bacteriology and cell biology, but is also responsible for a considerable amount of death and disease.

Treatment

The emergence of antibiotics is often attributed to Sir Alexander Flemings discovery of penicillin in the 1920s. However, the first drug resembling what we now define as an antibiotic was Sulfanilamide, used to treat streptococcal infections, although historians have shown that topical application of mouldy bread was used with some success as early as the classical times [141]. Today, a wide array of antibacterial agents makes up the list of available antibiotics. Broadly divided into either bactericidal or bacteriostatic, they are often further classified by the mechanism of action or chemical structure. The main bacterial targets of current antibiotics are the cell wall synthesis, protein synthesis, nucleic acid synthesis or the bacterial metabolism (Figure 4) [142].

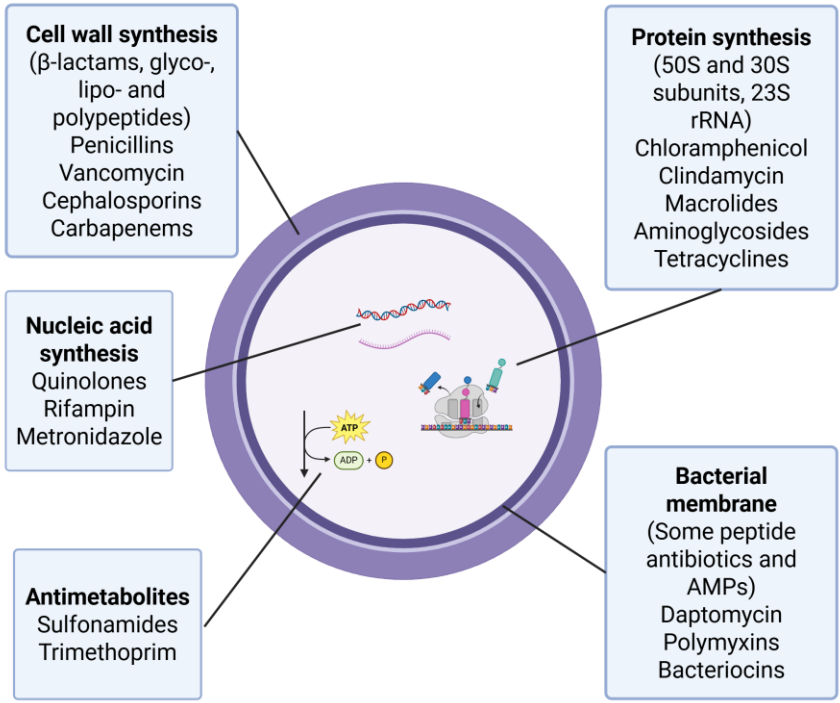

Figure 4. Overview of structures and processes targeted by antibacterial agents, with some examples.

The group of cell-wall synthesis inhibitors includes the **β-lactams** (penicillins, cephalosporins, carpapenems and monobactams), **peptide antibiotics** (vancomycin and bacitracins) and the special antibiotics acting against the unique cell wall of the mycobacteria (isoniazid, ethionamide, ethambutol and cycloserine). Related to antibiotics affecting the cell wall are daptomycin and the polymyxins. These antibiotics interact and permeabilize bacterial membranes, as opposed to inhibiting cell wall synthesis [143, 144]. Here must also be mentioned the antimicrobial peptides (AMPs), many of which target bacterial membranes. While only a few AMPs could currently be considered as antibiotics in a clinical sense, they are a central part of this thesis and thus described in detail in a separate section. Antibiotics responsible for protein synthesis inhibition include, but is not limited to, the

aminoglycosides (gentamicin, tobramycin), **tetracyclines, chloramphenicol, macrolides** (erythromycin) and **clindamycin**. Protein synthesis inhibitors target the subunits of the bacterial ribosome, or 23S rRNA. **Quinolones** (ciprofloxacin, levofloxacin and moxifloxacin), **rifampin** and **metronidazole** compose the inhibitors of DNA or RNA synthesis, and finally the antimetabolites include the **sulphonamides** and **trimethoprim** [145]. Furthermore, antibiotics can either be narrow-spectrum or broad-spectrum in their activity, and both types are found in all the above mentioned groups.

Antimicrobial resistance

In 1945, during his Nobel laureate lecture on the discovery of Penicillin, Sir Alexander Fleming finished with a warning:

> "But I would like to sound one note of warning. Penicillin is to all intents and purposes non-poisonous so there is no need to worry about giving an overdose and poisoning the patient. There may be a danger, though, in underdosage. It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body [146]."

Indeed, today, 80 years later, bacterial resistance not only to penicillin, but to all of the current groups of antibiotics available, is a major problem. For the past three decades, approximately one million deaths have been estimated to be directly caused by antimicrobial resistant (AMR) bacteria and five million are estimated to be associated with AMR infection, on a yearly basis. These numbers are forecasted to approximately double by 2050, if the issue is not properly addressed [147]. On a more positive note, the rate of mortality in children under 5 due to AMR-related sepsis has been reduced since 1990. This is the result of successful preventive methods such as increased sanitation and basic hygiene routines, highlighting the importance of preventing infection [147, 148]. In the elderly population, however, the rate of mortality has increased, and this is also the group where the forecasts predict the highest rates of mortality. Combined with the expected growth of those 70+ years of age during the next decades

[149], AMR bacteria is a looming threat we cannot leave unchallenged [150].

Mechanisms

Typically, acquired bacterial resistance to antibiotics stems from artificially imposed natural selection, i.e. the overuse of antibiotics not only in human health settings, but also veterinary and, to a large extent, in animal husbandry [151]. As mentioned, many bacteria possess intrinsic mechanisms and genes conferring resistance. This section focuses on acquired resistance and some of the mechanisms behind it, however, it should be noted that many of these mechanisms are often overlapping and could thus be considered as either intrinsic or acquired depending on the particular species being referred to. Mechanisms usually involve some of the following: bacterial modification of the target of the antibiotic, drug inactivation or modification via proteolytic or enzymatic activity and upregulation or downregulation of efflux and influx systems. These mechanisms are in turn often governed by alterations in the genetic code, that can be acquired from other bacteria, or through mutations resulting from adaptive pressure [152].

Although numerous examples exist, here follows some important examples of mechanisms and their basis, often found among the ESKAPE pathogens. One of the most frequently used groups of antibiotics are the β-lactams. Currently, there are over 7000 known β-lactamases [153], bacterial enzymes that bind, acetylate and then hydrolyzes β-lactam antibiotics [154]. First discovered acting against penicillins, β-lactamases have evolved parallel to the discovery of new β-lactam antibiotics, and there now exists several types of extended spectrum β-lactamases, capable of hydrolyzing modern generations of β-lactams, such as the cephalosporins, monobactams and in some cases the carbapenems [155, 156]. These enzymes are mediated by a vast number of genes, some of which can be found on bacterial plasmids and further disseminated to other bacteria [157, 158]. In what can be described as an arms race, the development of β-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam [159] for combination treatment, has given rise to hyper resistant strains

capable of inhibiting the inhibitors [160, 161]. Another mechanism of resistance to β-lactam antibiotics is alterations to the drug targets. The *mecA* gene coding for PBP2a has already been discussed in previous sections, and there are examples of other PBP-variants or mutations thereof [162, 163]. Mutations in DNA-gyrase confer resistance to quinolones and mutations of the ribosomal subunits or 23S rRNA affect the protein synthesis inhibitors. *P. aeruginosa* and *A. baumannii* excel at overexpressing efflux pumps, often chromosomally encoded but also transferrable via plasmids, and reduced expression of porins help other species to prevent update of the antibiotic [164]. As is the case with virulence factors promoting pathogenicity, mechanisms conferring resistance also often act in synergy with each other, and when combined with the formation of biofilm, can account for infections extremely difficult to eradicate.

Efforts to offset the increase in AMR involve infection prevention, enhanced diagnostics for finetuned treatment, limited use of antibiotics including in animal husbandry, social education, and of course research to find new and alternative treatments[165]. Unfortunately, the combined effect of efforts to reduce the use of antibiotics and conserving newly discovered antibiotics for last resort treatment has resulted in significantly decreased interest by the large pharmaceutical companies to invest in the research on novel antibacterial drugs [166, 167]. The World Health Organization has created a list where they group antibiotics based on their recommended use, i.e. first choice (**A**ccess), second choice (**Wa**tch) and last resort (**Re**serve), called AWARE. Together with recommended therapies for specific infections and causative pathogens, it aims to reduce the use of resistance-propagating antibiotics and promote the use of a carefully selected antibiotic (or avoid the use of an antibiotic all together). The list and tools provide excellent guidance and can be found at https://aware.essentialmeds.org/.

Wounds

The human skin is vital to our survival for a multitude of reasons and is a complex structure with numerous physiologic and biochemical functions. Consisting of the epidermis, dermis (with hypodermis),

and underlying subcutaneous fat of varying thickness, the skin is primarily a physical barrier preventing dehydration and providing protection against infection [168]. When this barrier is breached, pathogenic bacteria can enter the underlying tissue and cause an infection. These bacteria can be either opportunistic bacteria already residing on the skin or introduced via contamination of the traumatizing agent [169]. In a healthy human, bacterial infection following a skin injury is usually cleared by the innate immune system, although some soft tissue infections, such as erysipelas and cellulitis might require antibacterial treatment [170]. Under normal circumstances, acute wounds heal within a few weeks, through a highly complex process. The wound healing process can be subdivided into several overlapping phases, starting with hemostasis and inflammation, then followed by a proliferative phase, after which remodelling and maturation occurs [171]. Transition between these phases is dependent on a plethora of intricate molecular and cellular mechanisms, regulated by a multitude of growth factors, pro- and anti-inflammatory molecules, proteases and other cytokines [172], described in brief in Figure 5. Ultimately, closure of the wound is completed through contraction of the wound, usually followed by some degree of scar formation [173].

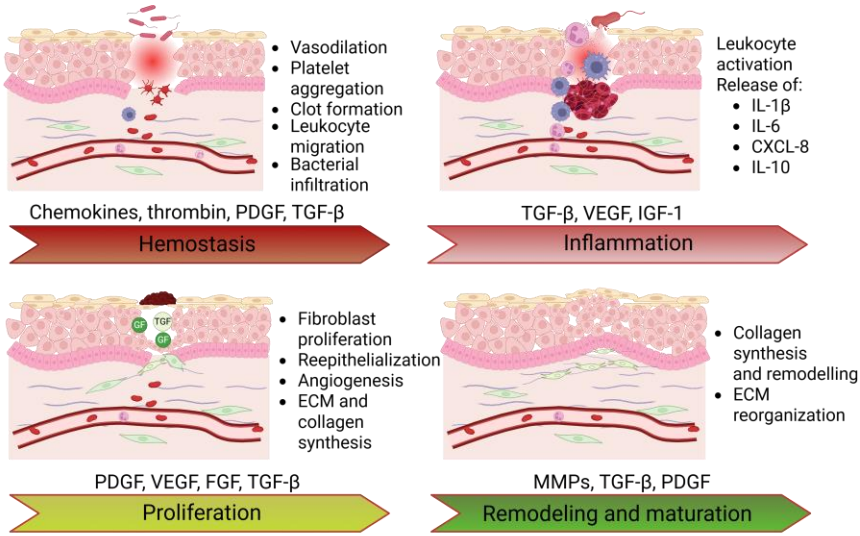

Figure 5. Normal wound healing. During **hemostasis**, blood vessels dilate, and platelets reach the injured site where they activate and begin forming a clot. Chemokines attract leukocytes, primarily neutrophils and macrophages, and bacteria can infiltrate. Once leukocytes reach the injury, **inflammation** commences mediated by proinflammatory cytokines, and infection is eradicated (under normal circumstances). This is followed by reepithelization and fibroblast **proliferation** mediated by growth factors, and **maturation** is completed via synthesis, remodelling and reorganization of collagen and ECM close the wound. Adapted from Yang et al. [174] and Gurtner et al. [175].

Host-pathogen interactions

If the skin is breached, bacteria can invade. There, they will be met by resident cells of the innate immune system such as macrophages, neutrophils and antigen-presenting cells (APCs) together with keratinocytes, also possessing innate immunity functions [176, 177]. Pathogen-associated molecular patters (PAMPs), derived from damaged bacteria within the tissue or presented by APCs, are then recognized by pattern recognition receptors (PRRs). Two well-known groups of PRRs are the ten different toll-like receptors (TLRs), found in the plasma membranes or on intracellular structures, and the nucleotide oligomerization domain (NOD)-like receptors, found in the cytosol

[178, 179]. In the context of bacterial infections, TLR2 and 4 are of particular importance. TLR2 recognizes PAMPs originating from gram positive bacteria, such as LTA and peptidoglycan, whereas TLR4 is important for the detection of gram negative species, being especially attracted to LPS [180]. Activation of TLRs and NOD-like receptors, with the exception of TLR3, triggers a signalling cascade resulting in the activation of the gene regulating nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) pathways [181, 182]. When activated through PRRs, the NFκB downstream signalling cascade unfolds via the canonical pathway, primarily involving the transcription factors p50 (also known as NF-κB1), RelA and c-Rel. p50 undergoes dimerization with RelA (p65) and c-Rel, and the resulting p50/RelA and p50/c-Rel dimers translocate to the nucleus, where they activate transcription of several pro-inflammatory cytokines and chemokines such, as IL-6, CXCL-8 and MCP-1 [183]. Three families of MAPKs are found in humans, namely the c-Jun amino-terminal kinases (JNKs), the extracellularly regulated kinases (ERKs) and the stress-activated protein kinases known as p38/SAPKs [184]. Upon activation, core kinases ($MAPK_3$, $MAPK_2$ and MAPK) are sequentially activated by phosphorylation, ultimately activating regulatory proteins that in turn modulate transcription factors in the cytosol or nucleus [185]. The resulting gene expression codes for factors involved in proliferation, differentiation, apoptosis and expression of proinflammatory cytokines and chemokines [186].

Non-healing wounds

Some wounds, however, fail to heal. While no universally accepted definition exists, wound failing to heal within 6-12 weeks are normally considered as chronic or non-healing wounds [187]. These pathological wounds are usually found in patients suffering from underlying comorbidity, such as diabetes, vascular insufficiencies, immunosuppression and cancer, in addition to increasing age and lifestyle factors such as smoking, obesity, malnutrition and immobilization [188]. As a consequence of the rise in incidence of non-healing wounds in recent decades, the associated socioeconomic burden and morbidity has significantly increased [189]. The pathophysiology stems from a combination of micro- and macro-vascular

ischemia, neuropathy and bacterial colonization which propagates the transition from an acute wound to a non-healing wound [190]. Non-healing wounds are arrested in a perpetual inflammatory phase, characterized by an increased presence of immune cells (primarily neutrophils) combined with dysregulated levels of proinflammatory cytokines and proteolytic enzymes [191-193]. Such conditions lead to increased tissue destruction, promoting bacterial colonization and biofilm formation, further impeding the healing process [194].

S. aureus and coagulase-negative staphylococci are normally the first pathogens to colonize a wound, however, non-healing wounds are seldom infected by only one species of bacteria. Apart from the above mentioned bacteria, *Enterococcus faecalis*, *Enterobacter cloacae* and *P. aeruginosa* (all belonging to the earlier mentioned ESKAPE pathogens) are commonly found in non-healing wounds [187]. The composition of the bacterial flora present in a chronic wound is highly dynamic, where the interplay between bacterial species combined with systemic medicinal efforts to eradicate them results in a constant selective pressure. This will promote the rise of virulent and resistant strains, further exacerbated by nosocomial spread of already highly virulent and resistant strains [195, 196]. Indeed, non-healing wounds are commonly infected by several different types of multidrug resistant bacteria, predominantly residing within biofilms [197, 198]. Current treatment regimens of non-healing wounds usually consist of careful cleaning followed by debridement and treatment with topical or systemic antibiotics, after which the wound is covered by a dressing [199]. Topical treatment is considered more effective than systemic and does not promote the development of resistance to the same extent, however there is yet no consensus on the best topical treatment options or regimen [194]. Development of a topical treatment with a broad-spectrum antibacterial agent also capable of combating multidrug resistant bacteria, combined with a modern bioengineered dressing, might therefore be a promising approach.

Antimicrobial peptides

A group of molecules that has seen increasing interest in the past few decades are the antimicrobial peptides. Either found as naturally

occurring in practically all types of life or synthetically produced, the heterogenous AMPs are typically small peptides in the double digit amino acid residue range, with some type of antimicrobial activity [167]. Targeting fungi, bacteria or viruses, or a combination, they are an important part of a host's innate immunity and can range from having a rather broad spectrum of activity, to a more niched, narrow-spectrum activity [200, 201]. AMPs are often amphiphilic and positively charged and primarily target biomembranes, although examples of other targets, such as membrane proteins or intracellular components exist [202]. Generally accepted as the mode of action of the majority of AMPs, membrane permeabilization is postulated to be facilitated by electrostatic binding of the AMP to negatively charged phospholipids on the outer leaflet, after which some variation of membrane damage is introduced, i.e. via the formation of pores or channels, leading to leakage of intracellular content and eventual lysis [203]. AMPs are easily soluble in water, and their structure normally falls within one of five categories or classes; amphipathic α-helices, β-sheets with or without disulfide bonds, a combination of α-helices and β-sheets, linear extended structures and finally loop or cyclic structures [204-206]. They are furthermore classified based on their origin (i.e. mammalian, microbial, amphibian etc.), their target of activity and eventual high content of specific amino acids such as proline, glycine or histidine [202]. They can have microbicidal effects, but many are also potent immune modulators and effectors of the immune system. Some AMPs are constitutively expressed, whereas others are inductively expressed upon stimulation with immunologic or inflammatory molecules, such as PAMPs and inflammatory cytokines, and are thus often referred to as host-defense peptides [200].

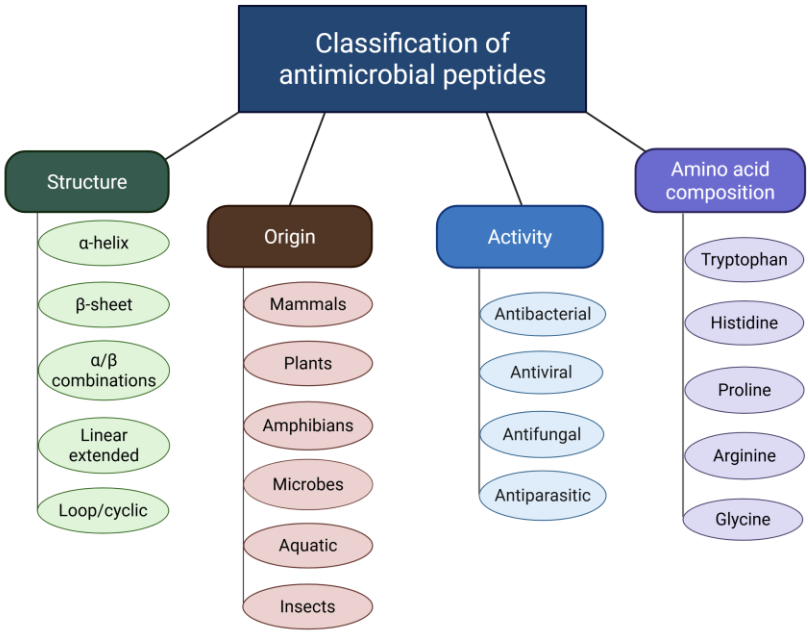

Figure 6. Classification of Antimicrobial Peptides. The classification of AMPs based on structure, origin, activity and high content of specific amino acids.

In mammalian cells, there are primarily two types of antimicrobial peptides produced, namely the cathelicidins and the defensins. Cathelicidins are expressed in a propeptide state, predominately found in granulae within immune cells but also in several epithelial cells and require proteolytic cleaving of a defining N-terminal segment to become active. This segment is also called the cathelin domain and is evolutionally conserved among the otherwise heterogenous group of cathelicidins [207]. While some mammals express several different cathelicidins, only one is found in humans, namely the well-researched host-defense peptide LL-37 (hCAP18). LL-37 adopts a α-helical structure and has microbicidal effects against bacteria [208], viruses and fungi [209, 210], while also modulating the immune response by upregulating pro-inflammatory cytokines and chemokines, and also acting as a chemoattractant for immune cells in itself [211, 212]. Similarly, the defensins are also initially expressed as

propeptides requiring proteolytic cleaving for activation, and are found in neutrophils, mucosa and epithelium [213]. Defensins are categorized as α-, β- or θ-defensins depending on disulfide bond localization between the β-sheets making up the core structure, and only the first two are found in humans. Contrary to cathelicidins, there are multiple different defensins found in humans, with a more specialized spectrum, although still broad-spectrum in the general sense [214]. While considered less potent in *in vitro* studies than LL-37, they nevertheless have antibacterial, antiviral and antifungal activity, and have been shown to kill bacteria via membranolytic mechanisms [215-217] or by disrupting cell wall synthesis by targeting lipid II [218, 219].

Bacteriocins

Another type of AMPs that can be found in the human body, however not produced by our own cells, are the bacteriocins. As the name implies, these are antimicrobial peptides synthesized and released by bacteria. First discovered in the 1940's [220], bacteria utilize these potent AMPs to compete for nutrients and space with other bacteria occupying the same environment. More potent than the AMPs found in eukaryotes, bacteriocins are produced by most of the currently known species of bacteria, highly diverse, and often narrow-spectrum, targeting related bacteria that are competing in the same niche or microenvironment, although examples of bacteriocins with broad-spectrum activity are also abundant [200, 221, 222]. Originally divided into two groups based on the presence or absence of the non-proteogenic amino acid lanthionine, the classification of bacteriocins have been constantly evolving since their discovery [223]. Most of the characterized and well known bacteriocins originate from gram positive bacteria, especially the lactic acid bacteria group (LAB), from which several highly potent bacteriocins have been isolated [224]. Bacteriocins from gram negative bacteria are poorly understood and not as well characterized, which in combination with indistinct nomenclature practices generates some confusion. Briefly, bacteriocins from gram negative species are referred to as colicins or microcins, which constitute a group of small (<5 kDa), highly diverse and primarily narrow spectrum bacteriocins. Hence, the word bacteriocin now primary refers to

antibiotic peptides produced by gram positive bacteria [225]. An overview of bacteriocin classification based on recent recognized classification and nomenclature is provided in Table 1.

Table 1. Classification of bacteriocins from gram positive bacteria*

Class	Subclass	Description
Class I – Lantibiotics (Contains lanthionine) Heat-stable <5 kDa	Ia	Lantibiotics
	Ib	Labyrinthopeptins
	Ic	Sanctibiotics
Class II – Non-lanthionine Heat-stable <10 kDa	IIa	Pediocin-like bacteriocins
	IIb	Two-peptide bacteriocins
	IIc	Cyclic bacteriocins
Class III Heat-sensitive >30 kDa	N/A	Large, heat labile proteins
Class IV	N/A	Large complexes containing lipids or carbohydrates

*Adapted from Singh et al. [226], Drider et al. [227] and Oppegard et al. [228].

Of special interest for this thesis are the class II b Plantaricins produced by the gram positive commensal bacterium *Lactobacillus plantarum* [229]. Encoded by genes located on the mosaic pln gene loci found among *L. plantarum* strains, plantaricins are potent inhibitors of several bacteria and viruses [230]. The focus in this thesis is on Plantaricin NC8 αβ (PLNC8 αβ) and synthetic derivatives of its' β-peptide (the C5 lipopeptides further discussed in the Results and Discussion section). The α and β subunit of PLNC8 consists of 29 and 34 amino acid residues, respectively, and require the combined activity of both subunits for full activity, although the individual peptides do exhibit some membrane activity, primarily the β subunit [231, 232]. PLNC8 αβ has been shown to inhibit several gram positive bacteria

[231, 233], the gram negative bacteria *Porphyromonas gingivalis* [234] and several species of *Salmonella spp.* [235]. Furthermore, recently PLNC8 αβ has also been shown to have antiviral activity against some enveloped viruses including SARS-CoV-2 [236]. Most evidence suggest that PLNC8 αβ permeabilizes negatively charged membranes, suggesting preference for prokaryotic membranes over eukaryotic, as the latter tends to localize negatively charged phospholipids to the inner leaflet, leaving the outer less reactive to electrostatic interactions [237].

Bacterial biomembranes are evolutionary conserved structures that are not as easily altered in response to AMPs, suggesting that resistance against these peptides might be less likely to develop [238, 239]. Furthermore, peptide drugs are easily modified to alter their characteristics, and methods advancing the rational design of synthetic peptides have improved, presenting advantages in the fight against AMR bacteria [240-242]. However, there are currently thousands of known AMPs, natural or synthetic, with proven antimicrobial activity *in vitro* [243, 244]. Why then, are there not thousands of novel antibacterial drugs being made available for clinical use? As with all drug development, time and money are of course limiting factors. However, there are other, more problematic reasons. Even though many AMPs have shown great effects *in vitro*, the translation to *in vivo* have been inefficient, and systemic administration of AMPs is problematic in regard to both toxicity and bioavailability, partly on account of peptides being reactive and easily hydrolyzed via proteolytic enzymes [245, 246]. Lately, increasing evidence suggesting development of resistance to AMPs have accumulated, and many bacteria have evolved mechanisms to evade AMPs [247]. Nevertheless, AMPs still present a promising approach in the struggle against bacterial resistance, especially considering potential localized or topical use.

In conclusion, the rise in antimicrobial resistance among already pathogenic bacteria is a severe threat to global health. Few new antibiotics are being developed, and despite efforts to control the use of antibiotics, morbidity associated with multidrug-resistant bacteria is forecasted to rise in the foreseeable future. Research on antimicrobial peptides as antibacterial agents has shown promising results in the

past few decades, but the transition towards clinical use has been challenging. Research focusing on finding clinical applications for AMPs, including engineering AMPs with enhanced properties to counteract some of the inherent limitations are thus of great relevance.

Hypothesis and aims

The overarching hypothesis of this thesis was that antimicrobial peptides could be utilized as topical treatments for bacterially infected wounds.

To evaluate this hypothesis, the specific aims include:

- To evaluate the potential of a select few AMPs in regard to their antibacterial activity, cytotoxicity and interaction and modulation of cellular signalling molecules and genes important to inflammation and wound healing.
- To design and develop *de novo* AMPs with enhanced properties to overcome some of the disadvantages inherent in AMPs.
- To design and conduct *ex vivo* and *in vivo* experiments to examine the effect of the enhanced AMPs in infected wounds to pave the way for clinical translation.
- To elucidate upon potential mechanisms of AMP resistance in the crucial gram negative pathogen *E. coli*.

Materials and methods

To answer the questions posed in the hypothesis, a combination of *in vitro*, e*x vivo* and *in vivo* approaches has been utilized.

Peptide synthesis and characterization (Study I, II)

Pre-synthesized PLNC8 αβ was purchased from GL Biochem (Shanghai) Ltd. The synthesis of the lipopeptides in study II was done using solid phase peptide synthesis with Fmoc protection. Rink Amide resin was used as support, and peptides were synthesized using an automated microwave peptide synthesizer (Liberty Blue, CEM). The Fmoc-protected amino acid residues were sequentially coupled using ethyl cyanohydroxyiminoacetate (Oxyma) and diisopropylcarbodiimide (DIC) as coupling agents for increased yield. After synthesis, Fmoc was removed by 20% Piperidine in dimethylformamide (DMF). Acetylated peptides were created by treating the resin-bound peptide with acetic anhydride in DMF (1:1), and lipidated by n-alkane acids combined with amidinium (HCTU) and dipropylethylamin DIEA in DMF. Trifluoroacetic acid (TFA) was used for final deprotection and removal of peptides from the resin support. Peptides were then concentrated in nitrogen and purity controlled. Finally, the identity of the peptide was confirmed utilizing a Maldi-ToF mass spectrometer (Bruker).

Determination of lipopeptide secondary structure and aggregation (Study II)

To determine eventual secondary structures and aggregative potential of the C5 peptides, circular dichroism (CD) spectroscopy and dynamic light scattering (DLS) was utilized. CD spectroscopy was used to determine the secondary structure of the *L*-enantiomers of 6-C5-N-Ile and 6-C5-N-Leu in solution or in the presence of lipid membranes. Experiments were conducted in a 1 mm light path quartz cuvette and evaluated in a Chirascan CD spectrometer at wavelengths between 195 and 280 nm. Lipopeptides (30 µM) were mixed with POPC:POPG 75:25 liposomes (1.2 mM) for 30 minutes, scanned three times and corrected against 10 mM, pH 7.4 PBS buffer. The

propensity of lipopeptide to self-assembly into larger structures was evaluated by DLS. Before measurements, lipopeptides were serially diluted from 100 μM to 0.01 nM, incubated for 5 min at 22 °C in a cylindrical glass cuvette immersed in refractive index matching toluene. Aggregation was then evaluated using an ALV/CGS-8F Platform (ALV-GmbH, Langen, Germany). The scattering intensity was obtained from an average of 10 consecutive 30 second long measurements.

Bacterial culture conditions (Study I-IV)

All species were kept in -80°C in LB broth and glycerol stock in a 1:1 ratio. Bacteria were then cultured in Luria-Bertani (LB) broth at 37 °C under constant orbital shaking at 250 rpm. Prior to experiments, the bacteria were washed twice in PBS, and bacterial concentration in CFU/mL was determined using McFarland standards. If not otherwise stated, the model bacteria used in this thesis are *Staphylococcus aureus* ATCC 12228 and *Escherichia coli* K-12 MG1655. All other bacteria within the thesis are clinical isolates of *S. aureus* (MSSA and MRSA strains), *E. coli* (non-ESBL or ESBL-producing strains), *E. faecium*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. cloacae complex* strains obtained from the Department of Laboratory Medicine at Örebro University Hospital. For specific details on the bacterial strains and eventual resistance profiles utilized in study II and III, the reader should refer to their respective studies.

Methods for bacterial susceptibility testing (Study I-IV)

The minimal inhibitory concentration (MIC) of the various peptide sequences and AMPs evaluated in the thesis was determined using the broth microdilution method. To determine bacterial concentration in a solution, (e.g. determination of minimal bactericidal concentration [MBC] from micro-broth dilution assays, homogenates from *ex vivo* and *in vivo* trials or supernatants from keratinocyte experiments), a ten-fold dilution series was prepared for each solution to be tested. 10 μL of each relevant dilution was then cultured in triplicates on an agar plate. LB or Mueller-Hinton agar was normally used, with the exception of the *S. aureus*-specific CHROMagar™ plates used for

quantification of bacterial load in *ex vivo* and *in vivo* wounds infected with *S. aureus*. After incubation overnight at 37°C, colonies were manually counted and concentration determined by multiplying the number of colonies with the dilution factor and dividing the product with the volume plated. The fluorescent nucleic acid stain SYTOX™ Green was used to evaluate bacterial membrane permeabilization. The dye binds to nucleic acids, but is unable to pass through intact lipid membranes, thus requiring membrane permeabilization to find its target, bind and fluoresce.

Checkerboard assay (Study I, II)

Synergistic, additive, indifferent or antagonistic relationships between PLNC8 αβ or 6-C5-N and various antibiotics were determined by checkboard assays. Two-fold serial dilutions of each AMP/Antibiotic pair were prepared longitudinally or horizontally in a 96-well plate and inoculated with 10^5 CFU/mL of bacteria. After overnight incubation, fractional inhibitory and bactericidal indexes (FIC and FBC respectively) were determined by adding the fraction of $MIC/MBC_{\text{AMP in combination}}/MIC/MBC_{\text{AMP alone}}$ with the fraction of $MIC/MBC_{\text{Antibiotic in combination}}/MIC/MBC_{\text{Antibiotic alone}}$. Synergism was determined if $\Sigma FIC \leq 0.5$, additive if between 0.5 and 1, indifferent when between 1 and 2, and antagonistic if ≥ 2.

Liposome model (Study I and II)

The hypothesized membrane activity of PLNC8 αβ and the C5 lipopeptides was evaluated in a liposome model. Liposomes mimicking either procaryotic or eukaryotic were used, and the permeabilizing ability of the AMPs was determined by measuring the amount of released fluorescent carboxyfluorescein (CF) dye which had been encapsulated within the liposomes prior to exposing them to the AMPs.

For preparation of liposomes, the lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-Hexadecanoyl-2-(9Z-Octadecenoyl)-sn-Glycero-3-Phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) and Cholesterol (Chol) were used. Prokaryotic-mimicking liposomes with a 75:25 ratio of POPC:POPG and eukaryotic-mimicking liposomes with a 65:5:30 ratio of

POPC:POPS:Chol were prepared by thin film hydration. Briefly, lipids were dissolved in an organic solvent (chloroform), mixed accordingly and then homogenized. After homogenization the solvent was evaporated with a nitrogen stream, after which drying was achieved by overnight incubation in a vacuum desiccator and a thin film obtained. The resulting lipid film was hydrated with a CF solution and then incubated for 10 min with orbital shaking. Unilamellar liposomes was obtained via 21 extrusions through a 0.1 µm filter membrane. Prior to experiments, the liposomes were cleansed from unencapsulated CF by filtration through a gel filtration column. A solution of liposomes diluted in PBS to 25 µM was incubated with the peptides at concentrations ranging from 10^5–10^2 µM in triplicates, and CF release was measured every 2.5 minutes over the course of an hour.

Microscopy (Study I-IV)

All brightfield and fluorescence microscopy was conducted using an Olympus BX41 microscope. In the SYTOX™ Green assays, $5x10^5$ bacteria were suspended in PBS and exposed to PLNC8 αβ or the C5 peptides for 5 minutes, after which images were captured at 525/50 Em. Scanning and transmission electron microscopy (SEM and TEM) was used to visualize alterations to bacterial morphology after exposure to the AMPs. All samples were fixed in 5% glutaraldehyde and 0.1M phosphate buffer and cut into sections with a thickness of approximately 50 nm. Sections were then analysed in an Ultra 55 field emission SEM (Zeiss, Oberkochen, Germany) and a Hitachi HT 7700 (Tokyo, Japan) for TEM. Analyses and generation of quantitative data from resulting micrographs were done using ImageJ software.

Determination of cytotoxicity (Study I-III)

Cytotoxic effects on human cells in the various studies were evaluated by assessing cellular viability through visual inspection via bright-field microscopy, determination of lactate dehydrogenase (LDH)-release, live image analysis using computer-assisted proliferation assays (Incucyte® and Livecyte™ equipment) combined with DRAQ7 nuclei

staining. Finally, the determination of haemolytic activity was conducted using fresh heparinized human blood.

HaCaT cell culture conditions and experimental setup (Study I and III

HaCaT keratinocytes were maintained in DMEM supplemented with 10% FBS at 37°C and 5% CO2. Cells were passaged every two days and experiments were conducted using cells in passage 5-15. On the day before experiments, cells were collected, counted and seeded into a 24-well plate and cultured to approximately 90% confluency. At the start of the experiments, cells were washed in PBS and received fresh medium supplemented with 2% FBS. If the cells were to be infected, they were inoculated with bacteria at a multiplicity of infection (MOI) of 0.1 and incubated for 1 hour. Peptides were then added to the wells to reach the desired concentrations, and the plates were incubated. At the experimental endpoint, microscopy photographs were taken, after which the supernatant was collected. An LDH cytotoxicity assay was performed directly after collection of the supernatants, and the remaining samples were frozen for further evaluation of cytokines and other signalling molecules.

Viability was assessed qualitatively by examining cellular morphology and confluence utilizing bright-field microscopy. Photographs were taken in one quadrant adjacent to the centre of the various wells. LDH is found within most cells and leaks out into the surrounding media if the cell is dead or have lost membrane integrity. Once released of the cell, it can be detected and quantified colorimetrically, using an enzymatic reaction ultimately leading to the formation of a red formazan product, measurable at 490 nm. Data was then plotted as relative to the control.

Haemolytic activity (Study II) was determined by adding peptides in various concentrations to the wells of a 96-well plate containing 15% erythrocytes suspended in PBS. Plates were then incubated for 1 h at 37 °C, after which supernatants were centrifuged and the absorbance at 540 nm measured in a plate reader. All experiments were repeated three times.

Real-time cell analysis (Study III and IV)

In study III and IV, we used an Incucyte® S3 Live-Cell analysis system to follow keratinocyte viability and bacterial growth over time. HaCaT cells were prepared as described above and seeded into 96-well plates. After reaching confluency overnight, the media was changed to DMEM free of phenol red, supplemented with 2% FBS and 1 µM DRAQ7. DRAQ7 stains nuclei in permeabilized cells and was used as an indicator for cytotoxicity. Bacteria (*S. aureus* or *E. coli*) with a plasmid containing a green fluorescent protein (GFP) gene was then allowed to infect the cells for one hour (MOI 0.1) prior to the addition of peptides and/or Gentamicin, if applicable. The plates were then placed in the Incucyte® S3 Live-Cell analysis system and monitored over 48 (study III) or 24 (study IV) hours. GFP and DRAQ7 fluorescence intensity (representing *S. aureus* or *E. coli* growth and keratinocyte cell death, respectively) was measured and presented as μm^2/image.

In the Livecyte™ proliferation assay (study III), primary human fibroblasts and keratinocytes were used. Briefly, the primary cells were isolated from excess skin obtained after routine reduction surgeries of healthy patients, performed at Linköping University Hospital in Sweden. Cells were then seeded in flat-bottom 96-well plates and treated with a two-fold serial dilution of C5 peptides from 25 to 1.56 µM. Each sample was prepared in triplicates. Cellular proliferation was continuously measured in a Livecyte™ 2 kinetic cytometer from Phase Focus Ltd, Sheffield, UK. Determined cell numbers were normalized to the untreated controls and presented as a proliferative index.

Quantification of intracellular and extracellular signalling molecules (Study I and III)

The keratinocyte expression of cytokines and chemokines (IL-1 IL-6, MCP-1 and CXCL-8), growth factors and matrix metalloproteases into the cell media supernatants previously described was assessed via enzyme-linked immune-sorbent assays (ELISAs) or the Magnetic Luminex® assay. Gene expression was evaluated via real-time polymerase chain reaction (RT-PCR) and determined according to the ΔΔCt

method. The potential involvement of the NFκB and MAPK pathways in study II were conducted by treating HaCaT keratinocytes with NFκB, JNK and ERK inhibitors.

Evaluation of C5 peptides in *ex vivo* and *in vivo* infected wound models (Study III)

The effects of the C5 peptides on infected wounds and wound healing were evaluated using a human *ex vivo* skin model and an *in vivo* porcine wound model, described below. Furthermore, several gel compositions for topical administration were evaluated, ultimately resulting in a Pluronics® F-127 hydrogel suitable for our purposes.

Porcine *In vivo* wound model

A porcine wound model was utilized to examine the effects of *L*-C5-Ile and *L*-C5-Leu on wound healing and eradication of bacteria. The peptides were dissolved in physiological saline and administered directly into the wounds two days post infection with *S. aureus*. Additional treatment administrations were given every two days over the course of the experiment. Before administering the treatment, three biopsy samples from the edges of each wound were taken for CFU quantification. At termination, one half of the circular wounds were resected for histopathological analysis. Samples for CFU quantification were homogenized and serially diluted, plated on *S. aureus*-specific CHROMagar™ plates. After incubation, CFU/gram was calculated. The histopathology slides were either stained with Hematoxylin and Eosin for reepithelialization assessment, or with a primary polyclonal IgG *S. aureus*-antibody followed by a secondary antibody together with DAPI nucleic stain, for visualization of *S. aureu*s in the tissue. All experiments were conducted on female pigs under veterinary supervision. Ethical approval was obtained from the regional animal ethics committee in Linköping (ID 1418).

Human *Ex vivo* wound model

Excess human skin was collected in sterile physiological saline during routine abdominal reduction surgeries under sterile conditions. The collection, method development and experiments were conducted under ethical approval Dnr 2023-03000-01. No information on patients was collected, and all material was destroyed after use. No genetic material was explored. Experimental procedures were conducted immediately after the collection of the skin. The *ex vivo* infected wound model was prepared and conducted in a LAF bench, using sterile equipment and adhering to basic hygiene routines, as follows: First, all subcutaneous fat was removed using a scalpel and a pair of scissors, leaving a flat piece of skin consisting of the dermis and epidermis. Second, the piece was rinsed in sterile PBS and placed on a sterile cutting board with the epidermal side facing up. The epidermis was air-dried, and then gently swabbed with 70% ethanol. Third, after all alcohol had evaporated, circular, 3.8 mm in diameter, burn wounds were created by applying gentle pressure with a soldering iron for three seconds. The wounds were spaced approximately 2.5 cm apart from each other in all directions. Fourth, each wound was cut out by resecting a 1.5x1.5 cm full thickness square biopsy, with the wound centred in the middle, and placed in a 6 well plate. 1 mL of pre-warmed (37 °C) DMEM media supplemented with 10% FBS was added around the square biopsy, without submerging the epidermis. Finally, the wounds were carefully inoculated with 10 µL of bacterial solution, using a circular motion, and the plate placed in an incubator (37 °C, 5% CO_2).

In study III, 10^6 and 10^5 CFU of *S. aureus* and *P. aeruginosa*, respectively, were inoculated and allowed to infect for 20 hours, after which treatment was administered as described in the article. At the chosen timepoints, a full thickness biopsy of the entire wound and 2 mm of the surrounding tissue was resected, using a 6 mm in diameter biopsy punch, placed in a pre-weighed Eppendorf tube, weighed, and then covered with 150 µL of PBS plus glycerol stock in a 1:2 ratio and frozen before homogenization. Homogenization was conducted by mechanical mincing using a sterile pair of ophthalmic surgery scissors. A tenfold dilution series of the homogenates were then cultured in

triplicates on agar plates, incubated at 37°C overnight, after which the bacteria were manually counted and CFU per gram calculated. An overview of the infected wound model is described in figure 7.

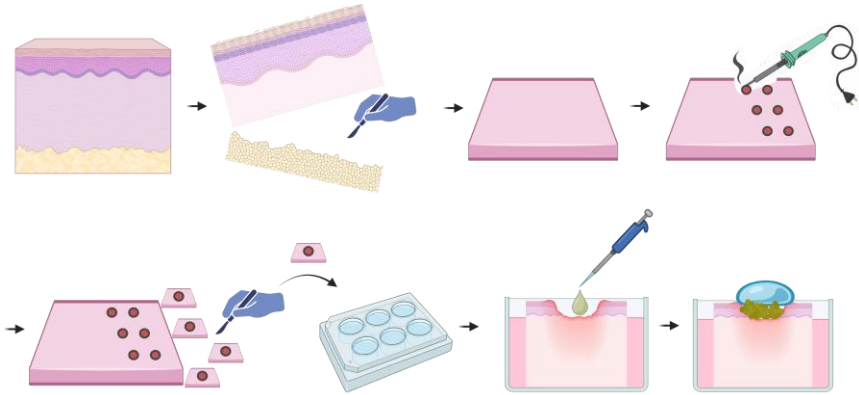

Figure 7. An overview of the *ex vivo* wound model. Subcutaneous fat was removed and a flat skin flap with the epidermis and dermis was created. Circular burn wounds were created using a soldering iron. Individual pieces were cut out and placed in a 6-well plate and supplied with 1 mL of cell culture media (DMEM+10% FBS), after which the wounds were inoculated with bacterial suspension. After 20 hours of infection, treatment was applied on top of the wound bed.

Hydrogel preparation

Pluronics® F-127 poloxamer was dissolved in deionized H_2O under cold conditions. A 20/80 w/v% solution of F-127 was prepared by adding F-127 to H_2O in a flat bottomed laboratory bottle placed on a magnetic stirrer located inside a refrigerator (4°C). F-127 was allowed to dissolve for 24 hours under constant stirring. After the poloxamer had fully dissolved, the whole solution was autoclaved for 15 minutes in 121°C. Powder-form *D*-C5-Ile was added to 1 mL of the autoclaved gel and vortexed until fully dispersed, resulting in two gel samples with concentrations of 4 and 10 mM respectively.

Statistical analysis

All statistical analysis was performed using GraphPad Prism version 9.2.0.332 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

Ethical considerations

In the works presented in this thesis, studies have been conducted on human blood, *ex vivo* human skin and animals. Haemolytic activity (Study II) was conducted on fresh heparinized blood collected from healthy volunteers, with the ethical permit Dnr 2015/543, approved by the regional ethical board at Örebro-Uppsala County. No information on the donors was collected. The isolation of primary human keratinocytes and fibroblasts was conducted under the ethical approval Dnr 2018/97-31. The *ex vivo* infected wound model on human skin was conducted under two ethical approvals: The development and optimization of the model was conducted under the ethical permit Dnr 2011/242, and the results in study III were generated using the finalized model that included the administration of treatment under the ethical permit Dnr 2023-03000-01. Excess skin destined for destruction was collected after routine abdominal reduction surgeries, and no information on patients was collected. No genetical material was analysed, and all samples and remaining tissue were destroyed after data acquisition. The porcine infected wound model was conducted under ethical approval from the regional animal ethics committee in Linköping (ID 1418).

Animal studies pertaining to drug discovery and development have long been considered an important step between *in vitro* studies and clinical trials in humans. As mentioned in the methods section, porcine skin and wound healing translates well to humans, and the species is often used in wound healing studies. Nevertheless, when conducting animal experiments, one must always consider the ethical component. Central to laboratory animal research ethics are the three Rs; replace, reduce and refine. Aiming to reduce the amount of animal suffering in animal research, the 3Rs ordains researchers to, if possible, replace *in vivo* experiments with experiments without animals (replace); reducing the number of animals used to strictly necessary numbers (reduce), and at all times consider animal well-being by using as humane methods as possible, while providing enrichment and making the animals as comfortable as possible (refinements). In this regard, the human *ex vivo* skin wound model can be used to fine-tune drug formulations, concentrations and treatment regimens

before conducting animal experiments, reducing the total amount of animals needed, and can thus be considered as a reducing factor.

Results and discussion

Summary of key findings

In **study I**, cytotoxic and immunomodulatory effects of PLNC8 αβ and *S. aureus* on the immortalized human keratinocyte cell-line HaCaT were evaluated. This was followed by examining the same parameters, as well as antibacterial effects, when treating *S. aureus*-infected HaCaT cells with PLNC8 αβ. Our findings show that PLNC8 αβ has a MIC of 12.5 μM and MBC of 25 μM against both *S. aureus* and MRSA. PLNC8 αβ alone is not cytotoxic for keratinocytes and induces an acute but short-lived expression of proinflammatory cytokines. Contrarily, *S. aureus* conferred significant cytotoxicity to keratinocytes and upregulated proinflammatory cytokines and gene expression via activation of NFκB and MAPK signaling pathways. Importantly, addition of PLNC8 αβ to infected keratinocytes significantly reduced the *S. aureus*-mediated inflammatory response and enhanced keratinocyte survival, suggesting a potential role for PLNC8 αβ in the treatment of wound infections.

Study II aimed to develop novel synthetic AMPs with improved antibacterial activity and a broader spectrum of targets, while simultaneously increasing stability and reducing production costs. By utilizing databases of known antibacterial amino-acid sequences, a short series of peptides with predicted antibacterial activity was synthesized. One sequence showed antibacterial activity, which was further enhanced by acetylation, lipidation, amino acid substitution and synthesis with *D*-amino acid residues, resulting in four C5 lipopeptide variants denominated *L/D*-6-C5-N-Leu and *L/D*-6-C5-N-Ile. These C5 lipopeptides exhibited inhibitory and bactericidal activity against both gram positive and gram negative species. Furthermore, the C5 peptides displayed a preference for bacterial membranes over eukaryotic and show low cytotoxicity and haemolytic activity in regard to human cells.

Several experiments were designed and conducted to further elucidate upon the mechanism of action of the C5 lipopeptides, and to evaluate their effectiveness as topical treatment for infected wounds in **study**

III. Here we used electron microscopy to show that C5 causes bacterial aggregation followed by membrane disruption. Additionally, the C5 peptides modulated MCP-1, MMPs and vascular growth factors of keratinocytes infected with *S. aureus* and furthermore promoted survival of infected keratinocyte *in vitro*. Moreover, the C5 peptides, especially the *D*-enantiomers, effectively killed *S. aureus* and inhibited *P. aeruginosa* in a human skin *ex vivo* wound model and increased reepithelialization in porcine wounds infected with *S. aureus*, *in vivo*.

The surprisingly unsuccessful attempts to use PLNC8 αβ or the C5 peptides to protect keratinocytes infected with gram negative bacteria *in vitro*, led to the experiments conducted under **study IV**. Here we show that *E. coli* can utilize extracellular divalent cations present in the surrounding media in physiological concentrations to evade both AMPs, most likely by incorporating them into their outer membrane thus driving the net charge of the membrane towards the positive. Furthermore, exposure to AMPs induced expression of OMVs and fibrous structures, possibly suggesting initiation of biofilm formation. This provides further knowledge upon the ingenuity of gram negative bacterial defence mechanisms against antimicrobial peptides.

Antibacterial and immunomodulatory effects of PLNC8 αβ

Found to be effective against both methicillin-sensitive *S. aureus* (MSSA) and MRSA, treating *S. aureus*-infected HaCaT keratinocytes with 12.5 and 25 μM of PLNC8 αβ impeded *S. aureus* growth and promoted keratinocyte viability for several days. PLNC8 αβ alone was not cytotoxic to keratinocytes at the examined doses. Furthermore, the increased expression of the pro-inflammatory cytokines IL-6 and CXCL-8 induced by the bacteria was effectively offset by the bacteriocin. Interestingly, the activation of *TLRs 2* and *4*, *PAR-2* and *c-Jun* genes was significantly increased by PLNC8 αβ, whereas *p50* and *p65* were not. The same genes, with the addition of *c-Fos, IL-1β*, *IL-6* and *CXCL-8* were activated in cells infected with *S. aureus*, although at more pronounced levels. The genes elevated by *S. aureus* were in turn suppressed in the presence of PLNC8 αβ. The effect on gene expression by PLNC8 αβ alone was rapid and short-lived, suggesting an acute effect that could possibly promote early pathogen recognition

by the keratinocytes. The use of a JNK inhibitor further emphasized the role of c-Jun via JNK in *S. aureus*-mediated inflammation, and in contrary to the lack of activation of the NFκB-associated genes *p50* and *p65*, the addition of a NFκB inhibitor demonstrated that NFκB was indeed involved as well. PLNC8 αβ also modulated expression of several MMPs and vascular growth factors related to wound healing, both when administered alone or as treatment for *S. aureus* infection. Furthermore, we show that PLNC8 αβ acts in synergy with the antibiotic Gentamicin, providing treatment options that might facilitate reduced doses of the antibiotic, thus providing a small but important piece in reducing the development of antimicrobial resistance. We found PLNC8 αβ to have potential as a topical treatment for *S. aureus*-related infections, however, some of the inherent disadvantages (such as the codependency of two peptides, comparatively large size and lack of efficacy against several clinically important gram negative bacteria), prompted the investigation into alternative options.

Identification, design and evaluation of novel antibacterial peptides

While our research on PLNC8 αβ suggests great potential for topical treatment of primarily gram positive bacteria, chronic wounds are typically polybacterial in nature [248, 249], and a broad-spectrum antibacterial agent capable of inhibiting both gram positive and gram negative bacteria would be a more suitable treatment option. Contrary to the important and sensitive balance of the microbiota residing in the gastrointestinal tract, where systemic treatment with antibiotics is associated with negative effects [250], a wound should ideally be free of any microorganisms. Any bacteria residing within a wound can thus be targeted indiscriminately without affecting the host negatively. PLNC8 αβ relies upon the complementary action of two peptides, making administration more complex, as well as increasing production costs. In light of this, we aimed to develop a novel, smaller, antibacterial peptide with a broadened spectrum.

Since we had observed some antibacterial effects using only the β chain of PLNC8, or smaller truncated segments of PLNC8 β [231], we chose the amino acid sequence of this peptide as a basis for the search of novel compounds. A list of segmentally separated peptide

sequences consisting of 16 amino acids was prepared from PLNC8 β, by increments of one amino acid residue towards the C-terminal for each sequence. These sequences were then scored based on their predicted antibacterial activity using two databases, namely AntiBP [251] by Lata et al. (http://www.imtech.res.in/raghava/antibp/), and ADAM [252] by Lee et al. (http://bioinformatics.cs.ntou.edu.tw/ADAM). After synthesis and *in vitro* testing, sequence #6 with the amino acid sequence LGIKILWSAYKHRKTI was shown to exhibit antibacterial activity against both *S. aureus* and *E. coli*. This cationic sequence contained approximately 45% hydrophobic, 30% basic and 25% neutral amino acids, and was predicted to adopt an α-helical structure conferring amphipathicty to the molecule (Figure 8).

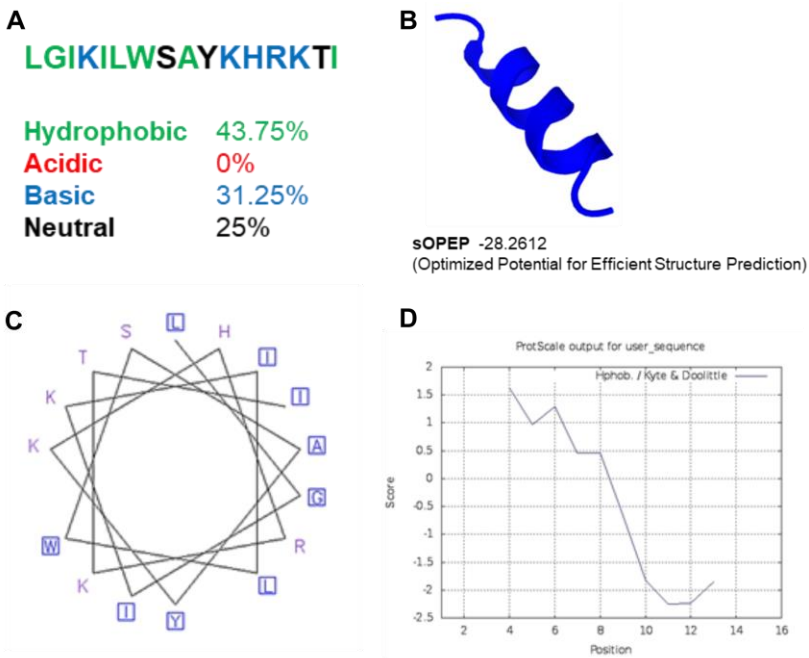

Figure 8. Characteristics of the optimized peptide sequence #6. A) The truncated peptide #6 is composed of 43.75% hydrophobic residues, 31.25% basic residues, and 25% neutral residues, with a net positive charge of 4.5. **B**) The peptide structure was predicted using the PEP-FOLD tool in the RPBS Wed Portal (https://bioserv.rpbs.univ-paris-diderot.fr/index.html). **C**) Distribution of amino acids in a helical wheel projection and **D**) hydrophilicity analysis using the Kyte-Doolittle plot.

This sequence was further optimized by acetylation and lipidation, the substitution of Isoleucine residues for Leucine residues, and synthesis using *D*-amino acids. Lipopeptides with fatty acids (FAs) comprised of 2, 4, 5, 6, 7, 8, 10 or 15 carbon atoms attached to the N-terminal were prepared. Interestingly, the best antibacterial activity was found in lipopeptides with 5-7 added carbons. Furthermore, determination of haemolytic and cytotoxic activity revealed that the peptides with a five-carbon FA tail was safest, which, combined with similar antibacterial activity as the six- and seven-carbon variants, contributed to the choice of the two peptides 6-C5-N-Leu and 6-C5-N-Ile for further development. The final sequence of 6-C5-N-Leu and 6-C5-N-Ile are CH3-(CH2)3-CONH-LGIKILWSAYKHRKTI and CH3-(CH2)3-CONH-LGLKLLWSAYKHRKTL, respectively, both with a formal net charge of +4.1.

Bacterial susceptibility testing of the selected AMPs (*L/D*-6-C5-N-Leu and *L/D*-6-C5-N-Ile) revealed both inhibitory and bactericidal activity against several pathogenic bacteria, including the ESKAPE pathogens and several strains of MSSA, MRSA and ESBL-producing *E. coli*, at micromolar concentrations (see table 4 in Study II). Circular dichroism spectroscopy revealed that both *L*-6-C5-N-Leu and *L*-6-C5-N-Ile predominantly existed in random coil conformation when in solution. This is common for many AMPs, that instead often adopt secondary structures when they encounter lipid membranes. Upon interaction with the POPC:POPG liposomes, a pronounced change in the secondary structure of the lipopeptides occurred, which indicated the presence of α-helical elements. However, further research on the possible secondary structure of C5 is warranted, especially considering the mix of amino acids present within the structure, offering both α-helix and β-strand preference, in addition to the presence of the structure breaking Gly and Ser residues [253]. In addition, DLS studies suggested lipopeptide aggregation in relation to membrane permeabilization, possibly by the formation of micelles, as per their amphipathic lipid structure. The critical aggregation concentration (CAC) of 6-C5-N was found to be 6.2 μM, which is close to the MIC and MBC of the same peptide versus *S. aureus* and *E. coli.* This suggest that permeabilization of bacterial membrane might be in part dependent on micelle-formation, although no clear evidence on whether

individual lipopeptides or micelles are the key factor in membrane disruption. Adak and colleagues have demonstrated that the four lipopeptides C_{16}-YKK, C_{16}-Ykk, C_{16}-WKK, and C_{16}-Wkk form micelles at concentrations below MIC values, suggesting a micelle-dependent antibacterial activity [254], and Chen et al. developed a series of aggregation-prone AMPs with enhanced efficacy [255]. However, micelles are instable, and could thus disassemble and insert into bacterial membranes upon contact, suggesting that the micelles are present primarily when the peptides are not in proximity to bacteria, possibly acting as a peptide reservoir [256]. Depending on the size of the micelles, one could presume that micelle formation might decrease the available soluble individual peptides, thus in essence reducing overall concentration. If they do disassemble upon contact with bacterial membranes however, the issue of micelle formation might be somewhat moot. Nevertheless, this further begs the question whether the peptides are consumed or not after interacting and permeabilizing the bacterial membrane, a question that we unfortunately lack the answer to at this point.

A multitude of natural or synthetic antimicrobial lipopeptides with varying amino acid sequences and lengths of FA tails are known, forming linear or circular structures in addition to larger supramolecular structures, and the length of the FA tail has been suggested to play a role in both antibacterial activity and eventual cytotoxicity towards eukaryotic cells [257, 258]. We found that conjugation 5-7 fatty acids conferred the best antibacterial activity, where the 5-FA C5 lipopeptides also showed the least haemolysis. Interestingly, haemolysis was low up to 5 fatty acids, but exponentially increased when adding 6 or more, where the highest examined number of 15 resulted in almost 40% haemolysis. Several studies have explored the relationship between FA chain length, antimicrobial activity and haemolysis, however there is yet not clear consensus on the optimal numbers of FAs or exactly how they influence these processes [259-261].

Mechanism of action of PLNC8 αβ and 6-C5-N

As discussed, the majority of AMPs act by permeabilizing bacterial membranes, and we hypothesize that both PLNC8 αβ and the C5 peptides are indeed also membranolytic. During the works of this thesis, several experiments aiming to elucidate upon the mechanism of action of the studied AMPs were performed.

In study I and II, SYTOX™ Green was used as an indicator of bacterial lysis. As mentioned, this fluorescent dye which binds to nucleic acids cannot penetrate intact membranes, and observed fluorescence will thus represent membrane permeabilization. MSSA and MRSA exposed to PLNC8 αβ for five minutes revealed substantial and rapid lysis. Likewise, *S. aureus* and *E. coli* exposed to the C5 peptides for five minutes resulted in similar results. Furthermore, the liposome model used in study II supplied additional evidence of the membrane permeabilising effects of the C5-peptides, while also showing a severalfold higher propensity for liposome models mimicking bacterial membranes over eukaryotic plasma membranes. These results also support the hypothesis that the negative charge of the bacterial membranes facilitate the initial interaction of C5. In study III and IV, electron microscopy revealed the rapid aggregation of both *S. aureus* and *E. coli* when exposed to *L*-6-C5-N. It is not clear whether this is a bacterial defence mechanism or a direct result of the activity of the lipopeptides. However, referring to the possible micelle formation of the lipopeptides discussed above one could hypothesize that micelles with the lipid moiety protruding outward could bind the membranes of multiple bacterial cells, contributing to the bacterial aggregation. Additionally, the fact that the *D*-enantiomers exhibit similar (or better) activity for membrane permeabilization suggests that they are indeed membrane-specific via electrostatic and hydrophobic interactions, and that they do not bind to any specific proteins or receptors on the bacteria.

Taken together, our results point towards bacterial membranes being the primary target for both PLNC8 αβ and C5, with a severalfold higher activity against prokaryotic than eukaryotic membranes. Both have rapid activity, disrupting membranes within minutes. Our results suggest that C5 and its variants approximately require an

additional 3-5 minutes to exert their lytic effects when compared to PLNC8 αβ. These extra minutes might possibly be concomitant with bacterial mitosis, as indicated by the abundance of mesosmal lipid structures observed adjacent to bacterial septa in electron microscopy of *S. aureus* exposed to C5 for 30 minutes. Contrarily, the permeabilizing effects of PLNC8 αβ appears to be more direct. Additionally, both peptides appear to induce a rapid bacterial aggregation prior to lysis, however, it is unclear if this is a direct effect of the peptides or rather a bacterial mechanism of defence. Bacterial auto aggregation (or flocculation) has been shown to act as a defence mechanism in different situations [262]. Several studies have proven the membrane permeabilizing activity of a plethora of AMPs (abundantly reviewed, e.g. by Raheem et al. [263]), often by pore-formation postulated to occur by a toroidal, barrel-stave, carpet or detergent-like model [264], but there are likely situational differences or additional effects that need to be elucidated. Using cryo-electron tomography, Chen et al. explored the mechanism of action of their AMP pepD2M by three-dimensional visualization. They show that although pepD2M harbours properties pertaining to pore-formation, the complete mechanism involved lipid removal followed by formation of lipid clusters attaching to the cell surface [265]. Similar lipid clusters have been observed in AMP-treated bacteria visualized by electron or atomic force microscopy [266-268], and some of the results from these studies resemble the SEM and TEM micrographs of *E. coli* exposed to PLNC8 αβ shown in study IV, suggesting a similar mechanism.

The preference for bacterial membranes suggests that eukaryotic cells would not be subjected to the permeabilizing effects of the AMPs. However, there are possibilities for other types of interactions conferring cytotoxic effects which must be evaluated. During the works of this thesis, we have observed that both PLNC8 αβ and the C5 peptides induce the production of pro-inflammatory cytokines, especially at higher doses. While this inflammatory response is more pronounced in the acute phase, it is important to evaluate further. Additionally, the C5 peptides, especially the variants comprised of *D*-amino acids and Leucine, were quite cytotoxic at higher concentrations. Nevertheless, concentrations well above MIC and MBC values for most bacteria were tolerable, and a topical treatment approach is

likely to be safe. Furthermore, both PLNC8 αβ and the C5 peptides were found to act synergistically or additively with several clinically important antibiotics such as Vancomycin, Gentamicin and Ciprofloxacin, suggesting a potential for a combined systemic and topical treatment. Finally, earlier studies on PLNC8 αβ showed no development of resistance in *S. aureus* when cultured at sub-MIC levels for 20 passages [231], and in study II it is demonstrated that *E. coli* and *S. aureus* remained susceptive to the C5 peptides after 30 passages of cultivation in sub-MIC concentrations.

C5 as treatment for infected wounds

The C5 peptides possess several traits making them suitable for topical treatment of wound infections. As discussed above, they display low cytotoxicity and haemolytic activity at doses severalfold higher than the MIC and MBC and furthermore exhibit broad-spectrum antibacterial activity against several pathogenic bacteria relevant to wound infections.

Modulation of factors important to wound healing

Keratinocytes are key players in wound healing. Evaluation of their response to *S. aureus* infection, with or without treatment with the C5 peptides, was thus considered an important part in the efforts to explore C5s potential as a topical antibacterial agent. The *D*-enantiomer and Leucine variants of the C5 lipopeptides induced more changes in the expression of keratinocytes than their Isoleucine and *L*-isoform counterparts, suggesting more reactive properties of the former. Infection with *S. aureus* primarily decreased the expression of MMPs 1, 2 and 10, and the vascular growth factors. Treatment with the C5 peptides at relevant doses (6.3 – 25 μM) primarily resulted in the counteraction of the inflammation induced by the bacteria, suggesting that the peptides might facilitate a return to a normal response. The delicate balance of cytokine, growth factor and proteolytic enzyme expression found in normal wound healing is commonly altered in chronic wounds, which is partly due to the degradation of growth factors by the upregulation of MMPs [269-272]. A topical agent modulating this balance towards a more favourable condition could thus be

beneficial in their treatment. It should be noted that these results are based on a single timepoint in what could be described as a reflection of an acutely infected wound, and the modulating effects of C5 needs to be evaluated over time before cogent conclusions can be drawn. Additionally, exposing primary fibroblasts to the C5 peptides promoted their proliferation. Fibroblasts are key players in wound healing and facilitating their proliferation could thus promote wound healing. However, this needs to be carefully evaluated as excessive fibroblast proliferation has been linked to the development of keloids [273].

Ex vivo and *in vivo* evaluation

To investigate the ability of C5 to eradicate bacteria in an infected wound, *ex vivo* and *in vivo* models were used. Porcine wounds were infected with *S. aureus* for 24 hours, after which they were treated with 1 mL of two concentrations (50 and 200 μM) of either *L*-C5-Ile or *L*-C5-Leu, once every two days. A total of three administrations were given during the 8 day long study. Simultaneously, work on developing a reliable *ex vivo* wound model was conducted. The results from the *in vivo* trials revealed a slight, but statistically insignificant, decrease in bacterial load, combined with a significant increase in epithelialization in treated wounds. Once the *ex vivo* wound model was established, initial data from infected wounds treated with *L*- and *D*-C5-Ile dissolved in H_2O revealed a reduction in *S. aureus* CFU by 2-3 orders of magnitude.

These initial *in vivo* and *ex vivo* experiments revealed issues such as evaporation and displacement of the administered peptide due to the low viscosity of saline or H_2O. Thus, efforts to improve the mechanical aspects of topically treating a wound with the C5 peptides were focused on the development of hydrogels as drug carriers. Several different alternatives for effective peptide delivery while simultaneously preventing displacement were examined. Eventually, a thermo-sensitive Pluronic® F-127-based hydrogel was developed and functionalized with C5 peptides (Figure 9).

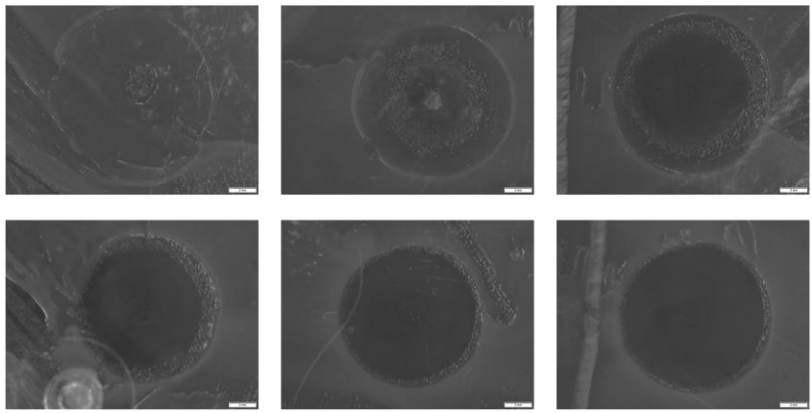

Figure 9. Development of F-127/C5 gels. The figure shows a dose-dependent increase in *S. aureus* inhibition by F-127 hydrogels functionalized with *L*-C5-Ile. **Top row:** Left: Control, Middle: 100 µM, Right: 200 µM **Bottom row:** 300 µM, Middle: 400 µM, Right: 500 µM. Scale bar is 2 mm.

Although substantially higher concentrations of peptide were required to reach sufficient effects, the hydrogel was chosen for its biocompatibility, ease of preparation and capacity for precise administrations. Furthermore, considering the increased stability of the *D*-amino acids, accompanied by the fact that *D*-C5-Ile yielded the best results in the pilot *ex vivo* experiments, further *ex vivo* experiments were conducted focusing on this mirror image. The final results show that one administration of *D*-C5-Ile dissolved in a F-127 hydrogel effectively inhibited *S. aureus* for 24 hours and decreased a highly virulent clinical isolate of *P. aeruginosa* several orders of magnitude (Figure 10). The results further show that the decrease in bacterial load is greater during the first 6 hours, suggesting that repeated administrations might provide a more rapid bacterial clearance.

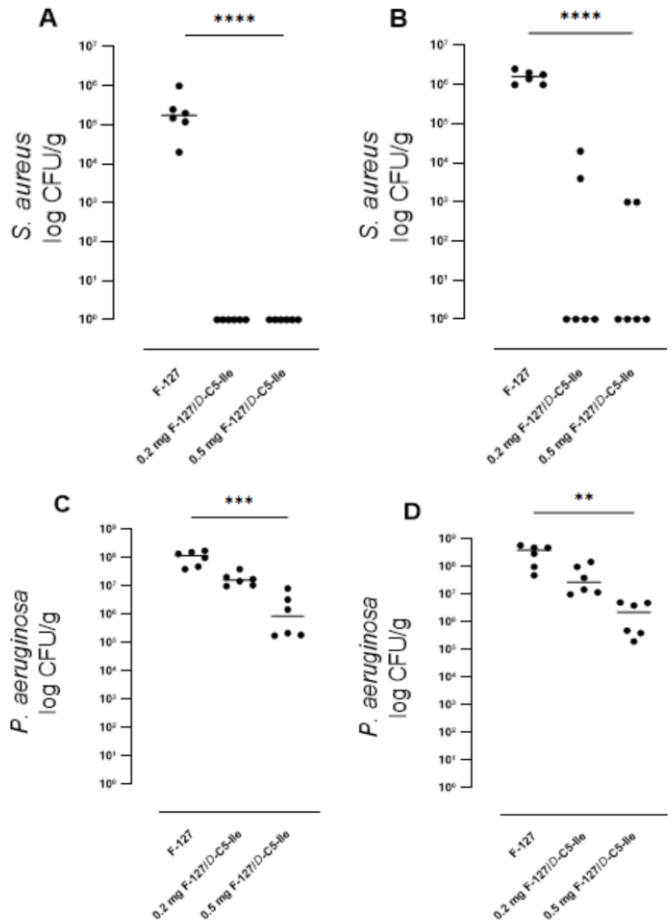

Figure 10. Ex vivo human infected wound model. CFU/g in wounds infected with *S. aureus* and treated with F-127/*D*-C5-Ile for 6 (A) and 24 (B) hours, and identically treated wounds infected with *P. aeruginosa* for 6 (C) and 24 (D) hours.

Higher concentrations of the C*5* peptides were required to reach inhibitory and bactericidal effects on *P. aeruginosa*, compared to most of the other bacteria examined in study II. *P. aeruginosa* produces an abundance of proteases, and indeed the D-enantiomers of C*5* were twice as effective as their counterparts. Furthermore, the *P. aeruginosa*

strain used in study III was a highly virulent clinical isolate, and evidence of biofilm formation was seen in the *ex vivo* wounds. This could explain why the treatment was not as effective against *P. aeruginosa* as for *S. aureus*. While the *ex vivo* model developed in this thesis is highly suitable for testing different formulations and finding suitable concentration ranges for treating various pathogens, it does not directly translate to an infected wound *in vivo*. It can however be successfully used as a proof-of-concept model, and to optimize the design of future *in vivo* studies. Unfortunately, we were not able to perform additional *in vivo* studies testing the *D*-C5-Ile/F-127 hydrogel, which is a limitation for this thesis.

Further limitations include the evaluation of eventual toxicity *in vivo*, i.e. if there is any systemic uptake or accumulation, and if so, how that could affect the host. Moreover, while infected wounds are problematic and impose a global health and socioeconomic burden, the need for alternative systemic treatment options is perhaps a more pressing concern. Little is known how PLNC8 αβ and the C5 peptides would affect the host if administered orally or intravenously. However, toxicology assessment of the intravenous administration of PLNC8 αβ in mice have been conducted by our team (data not presented in this thesis), where the no observed adverse-effect concentration was low (1.5 μM). While the exact causes of the detrimental effects observed are not clear, our hypothesis is that the peptide could interact with lipoproteins or cells within the plasma, resulting in the formation of microthrombi leading to embolization. Such studies have not been performed with the C5 peptides, but it is reasonable to assume that this AMP might also be reactive to molecules or cells residing within human blood. PLNC8 αβ is produced by *L. plantarum*, a bacterium associated with a healthy gut microbiome [274]. This suggests that oral administration of PLNC8 αβ could be safe and possibly help drive the balance of the gut microbiome towards a healthy composition. Most likely it would not be taken up systemically, preventing the use of PLNC8 αβ as a systemic treatment when taken orally. When considering the above points, topical administration appears to be the most promising route.

Evasion of AMPs by gram negative bacteria

The development of a novel AMP with activity against both gram positive and gram negative bacteria prompted the research on how the C5 peptides affected human cells infected with *E. coli* and *A. baumannii*. However, the C5 peptides were not able to fully counteract the cytotoxic effects exercised upon keratinocytes by these two bacteria. Considering that the observed C5 MIC and MBC values for *E. coli* and *A. baumannii* are almost equal to *S. aureus*, these results were surprising. Efforts to optimize the treatment by increasing peptide concentrations or administering multiple doses did result in prolonged bacterial inhibition but were not able to eradicate all bacteria (Figure 11). The surviving bacteria eventually grew into large enough numbers capable of killing all the keratinocytes within the 24 hour duration of the experiment.

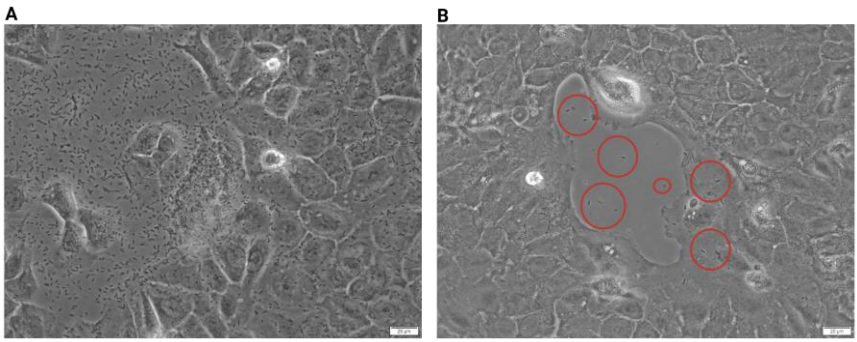

Figure 11. Figure 11 shows the presence of *E. coli* in the control (A) and wells treated with 20 µM *L*-C5-Ile, repeated every fourth hour (B) just after the third administration (12 hours post first administration). At this time, bacterial cells in B (red circles) were very few and could almost be counted manually. However, after 12 additional hours both the untreated keratinocytes (control) and peptide-treated keratinocytes were dead and wells were overgrown with bacteria.

The fact that we managed to inhibit almost all bacteria but that some managed to evade the treatment regardless of our efforts, led us to hypothesize over what the reasons may be. The gram negative OMVs immediately surfaced as prime suspects, however, as the bacteria

should be able to produce these already in the micro-broth dilution assay, we instead considered the differences between the two experimental setups. One major difference is of course the presence of human cells. However, *E. coli* are not intracellular pathogens, and complete eradication of keratinocytes by the bacteria took approximately 16 hours, during which time the peptides should have been able to destroy all the bacteria, as was the case with *S. aureus*. Another difference is the fact that *E. coli* thrives in a simple LB-broth, whereas human cells require a more complex medium such as DMEM for viability and growth. LB and DMEM both contain amino acids, vitamins and inorganic salts. However, the concentrations of magnesium and calcium in DMEM are approximately tenfold higher than in LB. This led us to design experiments where the effects of these divalent cations in relation to the ability of *E. coli* to evade C5 were investigated.

The addition of Mg^{2+} or Ca^{2+} prolonged the aggregation conferred by PLNC8 αβ and *L*-C5-Ile on the bacteria but completely abolished the lytic effects of the AMPs. Furthermore, electron microscopy revealed that PLNC8 αβ induces bacterial secretion of OMVs and the formation what appears to be fibrous structures that could possibly be Curli amyloids, suggesting initiation of biofilm formation. Additionally, the aggregation of the bacteria could further promote biofilm formation, as discussed above in relation to bacterial flocculation. Inorganic ions are important for the mechanisms involved in wound healing, and are thus likely accessible to bacteria colonizing a wound [275]. Considering the results of study IV, this could contribute to the pathogenesis of chronic wounds and their difficulty to treat. This hypothesis could be further explored with the aim of developing therapeutic adjuvants chelating such ions, which could assist AMPs and other antibacterial agents in inhibiting gram negative bacteria residing within a wound.

Conclusion and future perspectives

This thesis expands upon the field of antimicrobial peptides, offering insights in mechanisms of action and *de novo* design of novel antibacterial peptides. Additionally, functionalization and translatory efforts towards topical clinical application including *ex vivo* wound models that can reduce the use of animal research are presented. Furthermore, additional insight into the complex problem of methods of resistance and evasion to antibacterial compounds in gram negative bacteria is provided.

We conclude that PLNC8 αβ remains as an interesting candidate for further research particularly pertaining to skin infections with gram positive bacteria. Furthermore, we show that it is possible to engineer novel AMPs with enhanced stability and antibacterial properties using relatively simple means. Topical treatment with a hydrogel containing *D*-C5-Ile demonstrate great potential for the treatment of chronic wounds by reducing bacterial load and modulating important molecules related to wound healing. Additionally, we show that gram negative bacteria can utilize divalent cations as defence against positively charged AMPs, which adds important knowledge in the fight against gram negative infections.

To further elucidate upon these topics, several options are available. The *ex vivo* wound model can be used to test novel topical treatments and different methods of administration as well as formulations or functionalized wound dressings, before moving on to *in vivo* trials. Additionally, a polybacterial infection can be established to more closely resemble that of a chronic wound. Porcine wound models are highly suitable for evaluation of topical treatments and can be further utilized with the above mentioned improved administration methods. Additionally, murine models could be utilized to follow the systemic inflammation over time, as well as combining the topical treatment of the AMPs with systemic treatment of antibiotics.

With recent advances in *in silico* methods, including machine learning and AI tools, is it possible to identify and design drugs with very specific properties with a high throughput. Peptide drugs are very

suitable for such purposes, considering the ease with which they can be modified. However, any such drug candidates would still need to go through the due diligence of *in vitro* and *in vivo* testing before being considered for human use.

As mentioned, the precise mechanism of action of PLNC8 αβ or the C5 peptides is not fully understood, something that is also true for several other AMPs. Here modern microscopy methods, such as the cryo-electron tomography mentioned above, will provide a deeper understanding, especially in combination with *in silico* modelling and simulation.

As of today however, traditional wet lab practices remain an important cornerstone of medicinal research.

Acknowledgments

I am forever indebted to all those who have made this project possible. My apologies to anyone that I forgot to mention.

First and foremost; to my main supervisor, **Hazem Khalaf**. For giving me this opportunity, and for believing in me even when I have not. For being an inspiration in the field of science, creativity, curiosity and kindness. I am truly grateful to have been allowed to work together with you!

To my co-supervisors:

Torbjörn Bengtsson, thank you for sharing your knowledge, and for your encouragement. I greatly appreciate all your feedback and thoughtful comments, and your kindness.

To **Johan Junker** for your great insights into wounds and pigs. And of course all the beer and other antimicrobial beverages, and the music.

To Daniel Aili for the help with all the chemistry, and the great discussions and feedback.

To my fantastic colleagues in the research group, doctors **Abubakr Omer**, **Amani Musa** and **Sanjiv Kumar**. I've learned a lot from working together with you all and greatly appreciate the friendship and help throughout the years! Thanks to you all, the research group has been an example of how collaboration, curiosity and friendship fosters a fantastic work environment!

To everyone in the HEALiX team, it has been a fantastic experience to be part of a multidisciplinary team composed of such excellent researchers, I've learned a lot! And it has been really fun visiting castles and Spas throughout Sweden with you all. Special thanks to my coauthors **Elisa**, **Olof**, **Yağmur**, **Željana** and **Wasihun** for the great collaboration during these 5 years, and **Robert** for the peptides and bourbon guidance.

To all my colleagues at the School of Medical Sciences, in no particular order: **Madde**, **Antje**, **Rita**, **Kaya**, **Naveed**, **Ashok**, **Isak**, **Alexander**, **Liza**, **Assim**, **Fausto**, **Rosanne**, **Hung**, **Ignacio**, **Mulugeta**, **Wessam** and all others. It has been a pleasure working with you all. Thank you also to the administrators who've helped me throughout the years, **Liza**, **Susanne** and **Sofia**. To **Knut**, for your overwhelming positivity, and for teaching me how to make students cry.

To everyone at KFC, thank you for your kindness and help. A special thanks to **Lena**, **Taher**, **Seta** and **Therese** for your invaluable help with ordering, problem solving and guidance on using various machines. To **Anna Göthlin-Eremo** for the tutelage on confocal microscopy and the micro- and cryotome.

To **Maria Lerm**, **Blanka Andersson** and **Eva Danielson** at the Chemical Biology Consortium Sweden (CBCS), Linköping University Node, a national research infrastructure funded by the Swedish Research Council (dr.nr.2021-00179) and SciLifeLab.

To the Swedish Foundation for Strategic Research (SSF), for funding this PhD and the entire HEALiX project.

To my parents, **Bo** and **Ingela**, who introduced me to science and the wonders of nature. To my sister **Sofia**, for the discussions on science.

To my parents in law, **Tomas** and **Anna**, thanks for your support.

To my wonderful daughter **Linnéa,** for the joy you bring, and for teaching me about love and life.

And last but certainly not least, to my wife **Hannele Åhlén**, for your unwavering love and support, and for being such a fantastic mother to Linnéa. I could not have done this without you. I am eternally thankful that you have stood by my side, even though the last months of my writing this thesis was challenging for both of us, and apparently, I can be cranky at times when tired and stressed.

All figures in this thesis have been created in https://BioRender.com

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