UiT The Arctic University of Norway

Faculty of Health Sciences

Adhesion mechanisms and bacteriocins in *Staphylococcus haemolyticus*

New targets for the prevention and treatment of infections

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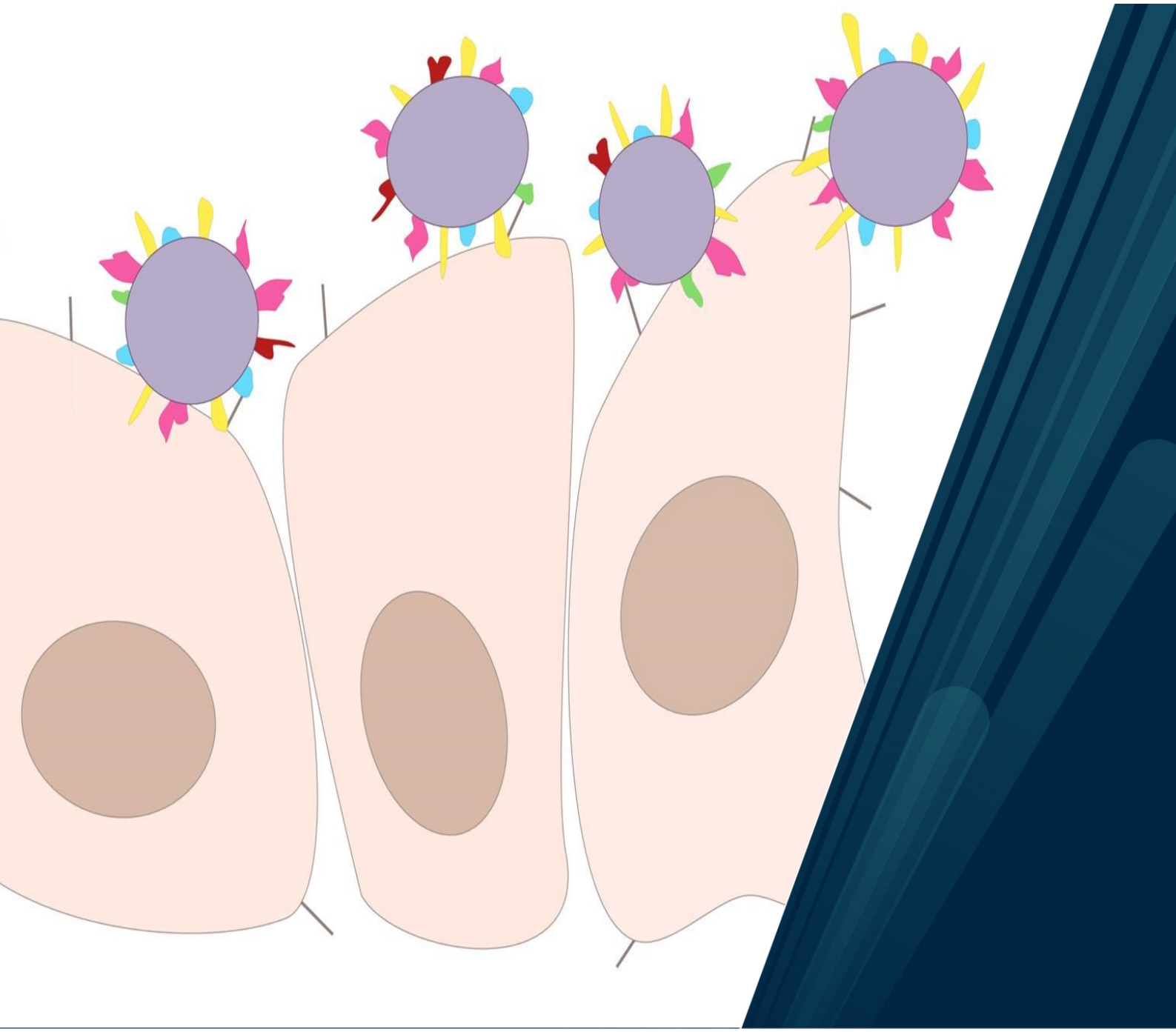

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// Runa //

List of papers

Paper I – Surface proteins

Runa Wolden, Maria Pain, Roger Karlsson, Anders Karlsson, Elizabeth G. Aarag Fredheim, Jorunn Pauline Cavanagh

Identification of surface proteins in a clinical *Staphylococcus haemolyticus* isolate by bacterial surface shaving

BMC Microbiology 2020

Paper II – A surface protein in detail

Runa Wolden, Martin O. Christensen, Jorunn Pauline Cavanagh, and Hermoine J. Venter

***Staphylococcus haemolyticus* SraP promotes binding to human cells**

Manuscript in preparation

Paper III – Bacteriocins

Runa Wolden, Kirill V. Ovchinnikov, Hermoine J. Venter, Thomas F. Oftedal, Dzung B. Diep, Jorunn Pauline Cavanagh

The novel bacteriocin romsacin from *Staphylococcus haemolyticus* inhibits Gram-positive WHO priority pathogens

Microbiology spectrum 2023

Abstract in English

Staphylococcus haemolyticus is a multifaceted bacterium that exists naturally in our microbiota but can also act as an opportunistic pathogen. Its ability to form biofilm, evade the immune system, and resist antimicrobials makes treatment challenging. This thesis aimed to deepen our understanding of its virulence factors and bacteriocin production to improve strategies for preventing and treating infections.

In **paper I**, we compared adhesion and biofilm formation of clinical and commensal *S. haemolyticus* strains, finding that clinical strains formed stronger biofilms. Surface proteins play a crucial role in adhesion and may serve as targets for new treatments. Using a surface shaving technique on a clinical strain, we identified several bacterial proteins after contact with human skin cells that could influence adhesion, biofilm formation, and immune evasion.

In **paper II**, we examined the SraP *S. haemolyticus* surface protein and its accessory secretion system. Mutants lacking SraP showed reduced adhesion and survival in human skin and lung cells, and decreased survival in blood. None of the strains triggered a strong immune response.

In **paper III**, we explored bacteriocin production in *S. haemolyticus*. We discovered a new bacteriocin, named romsacin after the Sami name for Tromsø, Romsa. Romsacin inhibited growth and eradicated biofilms of clinically relevant strains, including antibiotic-resistant *Staphylococcus aureus* and *Enterococcus faecium*.

In conclusion, our research has deepened the understanding of *S. haemolyticus* by exploring its bacteriocin production, virulence factors, and interactions with the human host. These insights could pave the way for more effective preventing and treatment strategies for *S. haemolyticus* infections and the potential therapeutic use of romsacin.

Samandrag på norsk (abstract in Norwegian)

Staphylococcus haemolyticus er ein bakterie med mange eigenskapar. Han finst naturleg i mikrobiotaen vår, men kan også vere opportunistisk patogen. Evna til å danne biofilm, unngå immunsystemet og motstå antibiotikabehandling gjer at behandling er utfordrande. Målet med avhandlinga var å utvide forståinga av virulensfaktorar og bakteriosinproduksjon hos bakterien for å finne betre måtar å forhindre og behandle infeksjonar på.

I **artikkel I** samanlikna vi adhesjon og danning av biofilm hos *S. haemolyticus* frå kliniske prøvar og friske vaksne. Vi fann at dei kliniske stammane danna sterkare biofilm. Overflateprotein speler ei avgjerande rolle i adhesjon og kan tene som mål for nye behandlingar. Ved å bruke ein teknikk som barberte overflata på ein klinisk stamme, identifiserte vi fleire bakterielle protein etter kontakt med menneskelege hudceller. Desse proteina kan påverke adhesjon, danning av biofilm og hjelpe til med å unngå immunforsvaret.

I **artikkel II** undersøkte vi overflateproteinet SraP hos *S. haemolyticus* og sekresjonssystemet som høyrer til. Mutantar utan SraP viste redusert adhesjon og overleving i hud- og lungeceller frå menneske, og dei overlevde dårlegare i blod. Ingen av stammane utløyste ein sterk immunrespons.

I **artikkel III** såg vi på bakteriosinproduksjon hos *S. haemolyticus*. Vi oppdaga eit nytt bakteriosin som vi kalla romsacin etter det samiske namnet på Tromsø, Romsa. Romsacin hemma vekst og fjerna biofilm frå klinisk relevante stammar, inkludert antibiotikaresistente *Staphylococcus aureus* og *Enterococcus faecium*.

Alt i alt har forskinga vår på *S. haemolyticus* gjort at vi betre forstår bakteriosinproduksjon, virulensfaktorar og interaksjonar med menneske hos denne arten. Desse innsiktene kan bane veg for å finne meir effektive måtar å førebygge og behandle *S. haemolyticus*-infeksjonar, i tillegg til å sjå på den potensielle terapeutiske bruken av romsacin.

Abbreviations

aacA	Aminoglycoside resistance gene
aphD	Aminoglycoside resistance gene
Asp	Accessory secretion protein
Atl	Autolysin
A549	Lung epithelial cells
BHI	Brain heart infusion
blaZ	Penicillin resistance gene
CFU	Colony forming units
EbpS	Elastin binding protein
Embp	Extracellular matrix binding protein
ENA	European Nucleotide Archive
FACS	Fluorescence-activated cell sorting system
Gft	Glycosyltransferase
HaCaT	Human keratinocyte cells
ica	Gene encoding PIA
IL	Interleukin
Isa	Lytic transglycosylase immunodominant staphylococcal antigen
LC-MS/MS	Liquid chromatography mass spectrometry
LPxTG	Sorting signal at the C-terminus of cell-wall anchored proteins consisting of Leu-Pro-x-Thr-Gly, with x representing any amino acid
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
mecA	Penicillin resistance gene
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
NAS	Non-aureus staphylococci
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PIA	Polysaccharide intercellular adhesin
PRR	Pattern recognition receptor
PSM	Phenol-soluble modulin

SasH	Mannosylglucosyl-3-phosphoglycerate phosphatase
SceD	Staphylococcus epidermidis D protein
Sdr	Serine-aspartate repeat protein
SecA2	Refers to the accessory secretion system for SRRPs
SecY2	Refers to the accessory secretion system for SRRPs
SEM	Scanning electron microscopy
SraP	Serine-rich adhesin for platelets
SRRP	Serine-rich repeat protein
SsaA	Staphylococcal secretory antigen
TIR	Toll/interleukin-1 receptor
TMT	Tandem mass tags
TNF	Tumour necrosis factor
TSB	Tryptic soy broth
VRE	Vancomycin-resistant *Enterococcus faecium*
WHO	World Health Organization

1 Introduction

Bacteria were the first life on earth and fossil evidence dates back 3.45 billion years [1]. The earliest animals evolved in environments already inhabited by bacteria. The lineage of *Homo sapiens* likely began at least 300,000 years ago [2], and there is an interplay with a variety of microorganisms in our bodies. In an adult man, the estimated number of bacterial cells is $3.8x10^{13}$ (0.2 kg) and human cells $3.0\ x10^{13}$ (70 kg) [3]. The microbial diversity is influenced by several factors, including disease, age, gender, and diet [4, 5]. Commensals dominate the bacterial flora and can protect against pathogens and disease [4, 6-9]. Bacteria occupy various parts of the body, including the mouth, stomach, gut, and skin. The skin is the largest organ of the human body and serves as a protective barrier from the external environment. A properly working skin microbiome is important for the skin to function [10].

Despite the interaction with commensals in our microbiota, bacteria also act as pathogens. They can for instance cause skin diseases, throat infections, sepsis, pneumonia, tuberculosis, urinary tract infections and food poisoning [9, 11-14]. Before the discovery of antibiotics there was a lack of effective treatment against bacterial pathogens. Surgical procedures and common bacterial diseases often resulted in severe outcomes. The first described antibiotic was penicillin. It was discovered by Alexander Fleming in 1928 [15], and the first person was treated with the drug in 1941 [16, 17]. Since then, antibiotics has saved millions of lives, and not only by treating infections. Antibiotics have enabled advances in medicine, such as major surgeries, cancer treatment, and organ transplants.

Today, antimicrobial resistance is a global health threat, and the clinical pipeline of new antimicrobials is dry. The World Health Organization (WHO) has declared that antimicrobial resistance is one of the top 10 global public health threats facing humanity [18]. Effective antimicrobials are important when treating infections and for the success of major surgery and chemotherapy. In 2019, 1.27 million deaths were attributed to bacterial antimicrobial resistance [19]. Some strategies to address the problem with antimicrobial resistance are development of vaccines and diagnostics, surveillance, reduced use of antibiotics in humans and agriculture, targeting host factors, combination therapies and development of new antimicrobial drugs [11, 18, 20].

The work in this thesis is primarily centred on *Staphylococcus haemolyticus*. The bacterium is naturally found on skin and mucous membranes but also acts as an opportunistic pathogen. It can cause disease in humans and animals, and its infections can be challenging to treat due to biofilm formation and resistance to antibiotics. Our research group has amassed a comprehensive collection of *S. haemolyticus* strains, encompassing both those isolated from clinical settings and those derived from healthy individuals [21-23]. Despite its clinical relevance, the available information about *S. haemolyticus* is sparse. Understanding the interactions between host and microbe is vital for the effective treatment of infections. Our project seeks to expand the knowledge about the virulence and antimicrobial bacteriocin production of *S. haemolyticus*.

1.1 *Staphylococcus*

1.1.1 Classification and characteristics

Staphylococci are a diverse group of Gram-positive bacteria that exhibit facultative anaerobic metabolism and are non-motile and non-spore-forming. Initially characterized by Rosenbach [24], these bacteria are classified within the family *Staphylococcaceae*, order *Caryophanales*, class *Bacilli*, and phylum *Bacillota*. As of January 22nd, 2024, the genus *Staphylococcus* comprises 66 validated species [25]. Under the microscope, staphylococci appear spherical, typically ranging in size from 0.5 to 1.5 µm in diameter. They are known to arrange in pairs, short chains, or grape-like clusters as shown in **figure 1**. The cell wall is composed of peptidoglycan, teichoic acid, and various proteins. While most staphylococci do not have a capsule [26], certain strains are known to produce one. Capsule production is an important virulence factor of *Staphylococcus aureus* [27], but can also be found in e.g. *S. haemolyticus* and *Staphylococcus epidermidis* strains [23, 28]. Staphylococci generally test positive for catalase, negative for oxidase, can tolerate high salt concentrations (10 % NaCl), and thrive in temperatures ranging from 18 to 40 °C [26].

Many species of staphylococci are commensals, residing harmlessly on the skin and mucous membranes of humans or animals, though some are opportunistic pathogens. They can also be sourced from animal products such as meat and dairy, as well as from environmental sources including fomites, soil, dust, and water [26].

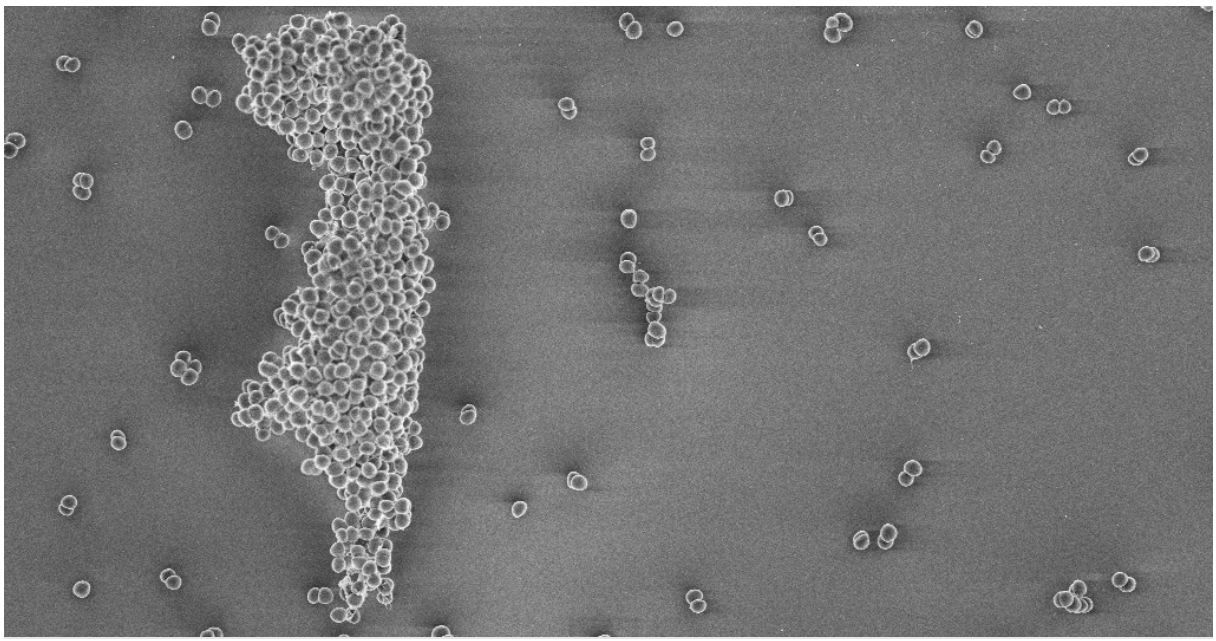

Figure 1 *- Scanning electron microscopy of S. haemolyticus (2,220×magnification). Photo: R. Wolden*

Staphylococci have traditionally been categorized into two groups based on their ability to produce coagulase, an enzyme that induces the fibrin of blood plasma to clot. *S. aureus* is the most clinically significant species within the coagulase-positive group [26]. However, some *S. aureus* strains are coagulase-negative, and there are also coagulase-positive staphylococci strains that are not *S. aureus*. I therefore use the term non-aureus staphylococci (NAS) to encompass staphylococci that are not *S. aureus* [29]. NAS are predominantly associated with the normal bacterial flora of humans but are also known as opportunistic pathogens. They can share similarities but are also found to be highly diverse [30]. Notable NAS species that are frequently associated with human disease include *S. epidermidis*, *S. haemolyticus*, *Staphylococcus lugdunensis*, *Staphylococcus saprophyticus*, *Staphylococcus capitis*, and *Staphylococcus hominis* [26, 31].

1.1.2 Clinical significance

Staphylococci can be harmless commensals of the human body but can also cause disease. The pathogenicity varies across species, though *S. aureus* is often considered a pathogen and is notorious for its association with infections and a robust array of virulence factors [26, 32]. Approximately 25-30% of humans are carriers of *S. aureus*, and these individuals have a higher likelihood of developing infections caused by their own carrier strains [33]. NAS were historically regarded as innocuous inhabitants of the human microbiota. However, their frequent implication in diseases, especially within hospital settings, has shifted this perception

[31]. There is an interaction between the host and the bacterium, and NAS pose an elevated risk to patients with weakened immune systems, including preterm infants and patients undergoing cancer treatment [34]. Although NAS can infect animals, this section will solely address their effect on humans.

The pathogenic nature of NAS is primarily attributed to their propensity to form resilient biofilms on medical devices, including catheters, prosthetic joints, and heart valves [34]. These biofilms present a challenge in clinical treatment due to their inherent resistance to antimicrobial agents. Infections can range from benign skin conditions to more severe cases like bacteraemia, endocarditis, and device-related infections [35]. The clinical significance of NAS is closely linked to advancements in medical technology, marked by a rise in the utilization of implanted medical devices and an expansion in the application of immunomodulatory therapies [31]. Vulnerable patient groups, such as elderly, preterm infants, and multimorbid patients, face a heightened risk of NAS infections [31, 36].

Between April 2018 and March 2023, there were 2,460 monomicrobial and 1,298 polymicrobial surgical site infections in the UK [37]. For the monobacterial deep or organ and organ/space surgical site infections, NAS was the second most causative microorganism after *S. aureus* in 4 of 6 categories. This included hip replacement (21.1%), knee replacement (23.4%), spinal surgery (18.6%) and coronary artery bypass graft (26.9%). For repair of neck of femur, NAS was the most causative agent (28.4%), and for large bowel the rate was low (4.2%). For monobacterial superficial surgical site infections, there was a smaller proportion caused by NAS, except for spinal (25.4%) and coronary artery bypass graft surgery (31.8%). For the last category, NAS was the most causative bacteria [37].

In a retrospective study (2016-2022) of NAS bloodstream infection in adults from a clinic in USA, 49/247 patients (19.8%) had infective endocarditis [36]. During a 10-month period in a Danish hospital, 65/585 (11.1%) of Gram-positive cocci bacteraemia were attributed by NAS. In the same period, there were 5 endocarditis caused by NAS [38]. In a nationwide Danish study, the prevalence of infective endocarditis with NAS bacteriemia was 8.1% when two blood culture sets were included [39]. Of 1760 patients with positive blood cultures over a 10-month period in a Saudi hospital, 208 patients had at least one positive blood culture with NAS [40]. Seventy-five were associated with infection, and the rest were considered contaminants. NAS have also been implicated in meningitis cases, where underlying conditions include diabetes

mellitus, liver cirrhosis, native valve endocarditis, hydrocephalus, alcoholism, and brain tumours [41]. Of 22,930 positive blood culture isolates in Norway in 2022, NAS accounted for 21.3%. However, the report did not distinguish between contaminants and clinically significant isolates [11].

Preterm infants (<37 weeks gestation) have a weak immune system, making them more susceptible to infections [42]. In the period 2009-18, 5296 live-born very preterm infants (<32 weeks gestation) were admitted to neonatal units in Norway, and 80% of the infants received antibiotic therapy [43]. Of the 493 cases of late-onset sepsis (sepsis after 3 days of life) in the nine-year period, 45% were caused by NAS. Late-onset sepsis is sepsis that occur after three days of life, and early-onset sepsis occur within the first three days of life.

The most significant NAS pathogens are *S. epidermidis*, followed by *S. haemolyticus* [31, 44]. Both species are linked to catheter-related bloodstream infections [45-48], and medical implant or prosthetic infections, such as prosthetic valve endocarditis [49-51]. Other NAS associated with disease are *S. capitis* and *S. hominis* [47]. In addition to primary infections, NAS can cause secondary bacteraemia in patients with moderate and severe viral pneumonia [52, 53].

There are examples of NAS species that can cause infections in otherwise healthy hosts. *S. saprophyticus* is a common cause of urinary tract infections, predominantly affecting sexually active young women [34, 54-56]. *S. lugdunensis* has a higher virulence potential compared to other NAS, and its infections can mimic those caused by *S. aureus*, such as endocarditis [36, 57, 58]. In fact, the odds for infective endocarditis with *S. lugdunensis* can be 13 times higher after blood stream infection when compared to other NAS [36].

1.1.3 *Staphylococcus haemolyticus*

This work mainly focuses on *S. haemolyticus* [59]. It is recognized as a skin commensal, but the organism also inhabits the gut [60], and acts as an opportunistic pathogen in humans and animals [11, 21, 31]. The name *S. haemolyticus* is derived from its haemolytic activity, which can be attributed to the production of phenol-soluble modulins (PSMs) [61]. The combination of immune evasion and antibiotic resistance within biofilms in addition to multi-drug resistance makes *S. haemolyticus* infections particularly challenging to treat [23, 62-66].

Bacteria can be organized into evolutionary lineages through genomic analysis, enhancing our understanding of their relationships and helps identify traits such as pathogenicity and antibiotic resistance across different groups. Multi-locus sequence typing categorizes bacteria based on a predefined set of conserved genes [67-69], whereas whole-genome sequencing enables a more detailed classification [23]. Phylogenetic reconstruction of 169 whole-genome sequenced *S. haemolyticus* isolates grouped them into six clades, where clinical and commensal isolates were distributed into different clades [23]. Distinct genetic signatures in the clinical strains were linked to their ability to thrive in the hospital setting [23]. A study by Soeorg et al. found that preterm neonates get colonized in the gut and skin by *S. haemolyticus* originating from the hospital environment, where the majority carried *mecA* resistance genes [70]. Breast milk was rarely a source of neonatal *S. haemolyticus* colonization [70].

Despite *S. haemolyticus* being a significant opportunistic pathogen, there is a scarcity of information on it, which necessitates further research. Subsequent sections will delve into pathogenic traits of *S. haemolyticus* in greater detail.

1.2 Pathogenicity

1.2.1 Surface proteins and host interaction

Staphylococcal surface proteins interact with host cell receptors (biotic), other bacteria, soluble macromolecules (such as fibrinogen, fibronectin and collagen) or abiotic surfaces (such as catheters and medical implants) and they are important for virulence and biofilm formation [48, 71-73].

Surface proteins can be associated with or attached to the bacterial surface (**figure 2**). Surface proteins covalently attached to peptidoglycan are referred to as cell wall-anchored proteins, and the array differs among staphylococcal species and strains [74-77]. All cell wall-anchored proteins contain a Sec-dependent signal sequence at the N-terminus and a wall spanning region and a sorting signal at the C-terminus. The sorting signal consists of an LPxTG (Leu-Pro-x-Thr-Gly, with x representing any amino acid) sortase cleavage motif, a hydrophobic domain, and a sequence of positively charged residues [71, 78]. Some cell wall-anchored proteins can bind to more than one host factor, and one host factor can be bound by several different bacterial surface proteins [34]. Some binding partners are still unknown. *S. aureus* may display up to 24 cell wall-anchored proteins [75], while *S. epidermidis* has a smaller repertoire [74]. The

knowledge about *S. haemolyticus* surface proteins is less comprehensive. Our research identified 19 proteins with predicted LPxTG motifs from the genome sequence of a single *S. haemolyticus* strain [79]. Among these, seven were classified as adhesion proteins and six had unknown function [79]. After exposing the bacteria to human keratinocytes and performing bacterial surface protein shaving, we identified five proteins with LPxTG motifs, of which three were adhesins [79].

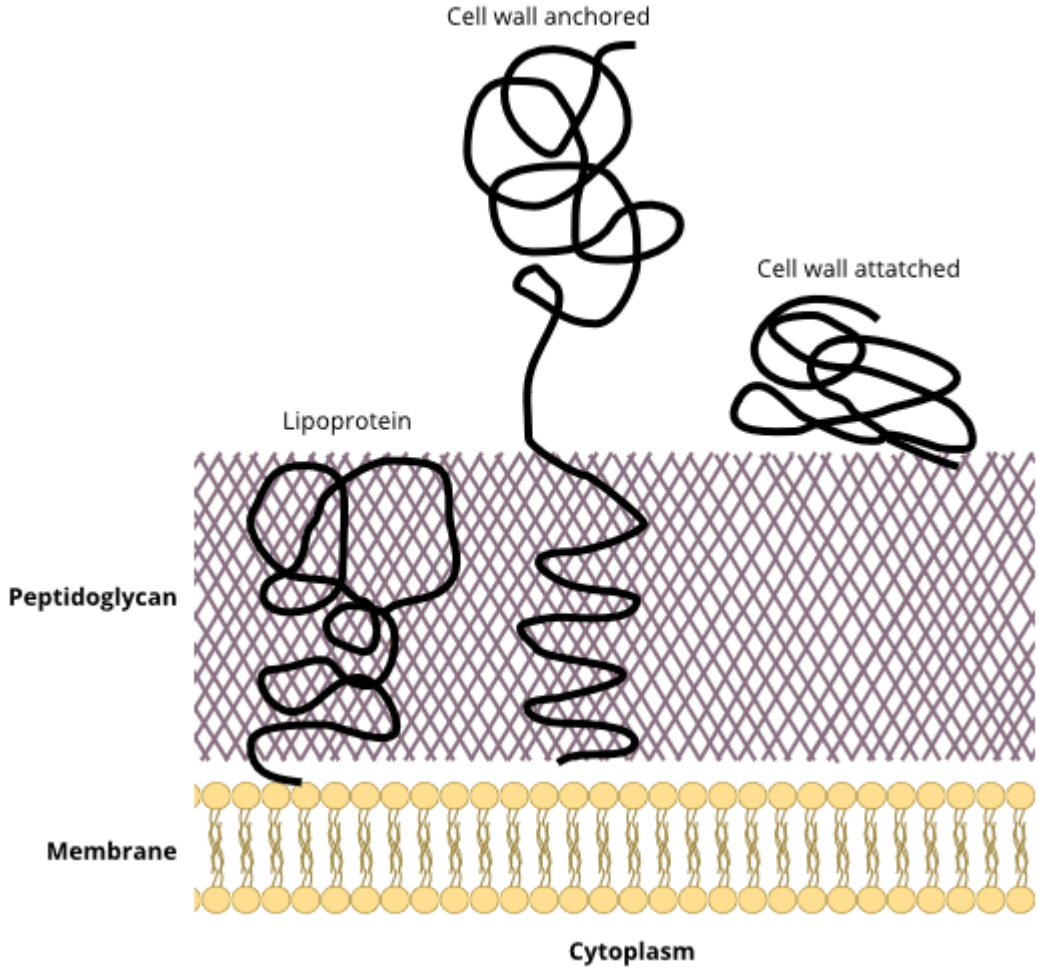

***Figure 2** – Bacterial surface proteins: Lipoprotein, cell wall anchored, and cell wall attached.*

The most prevalent of the cell wall-anchored proteins are the ones belonging to the family of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [34, 71]. The MSCRAMMs share a similar structure and a mutual mechanism for ligand binding, facilitated by two contiguous subdomains with IgG-like folds [71]. One approach for MSCRAMM ligand-binding is with the dock, lock, and latch mechanism [80]. The protein transitions from an open apo form that docks the ligand, to a closed form that locks the ligand before stabilizing the closed conformation in the latch [71, 80, 81]. The binding to ligands in this manner can be mechanically as strong as a covalent binding [82]. Many typical

MSCRAMMs contain serine-aspartate dipeptide repeats [83], and they have also been described in *S. haemolyticus* [79, 84].

Another type of cell-wall anchored proteins are the serine-rich repeat proteins (SRRPs) [85]. They are well described in streptococci [85], but are also found in staphylococci [23, 79, 86, 87]. SRRPs have a structure that includes an N-terminal signal peptide, non-repeat adhesion domain, long stretches of glycosylated serine-rich repeats, and a C-terminal LPxTG cell wall-anchoring motif [85, 86, 88]. The SRRP ligands vary between bacterial species, and not all ligands have been identified [88]. A dedicated accessory secretion system relocates SRRPs to the bacterial surface [85, 89, 90]. The accessory secretion system includes SecA2, SecY2 and three to five accessory secretion proteins (Asps) [90]. The serine-rich adhesin for platelets (SraP) in *S. aureus* is a SRRP which can bind to human platelets and is implicated in infective endocarditis [86, 91]. Pain et al. discovered a SraP homolog in 97% of clinical *S. haemolyticus* isolates, but only in 48% of commensals [23].

1.2.2 Biofilm

Infections caused by *S. haemolyticus,* or other NAS are often associated with biofilm formation, caused by their ability to attach to medical devices or host tissue. Staphylococci use a variety of mechanisms to adhere to abiotic and biotic surfaces, like physiochemical forces or production of surface proteins [34]. Surface proteins can bind to specific components of a medical device or host tissue. The interaction between the surface proteins and host mechanisms is crucial for the establishment of infection. Biofilm is a group of aggregated bacterial cells surrounded by an extracellular matrix. The aggregates can be single-species or multi-species. Biofilms can be attached to a surface but are also observed as non-surface-attached aggregates. The biofilm life cycle can be divided into three main stages: aggregation, growth and disaggregation [13], as shown in **figure 3**. During the aggregation stage, the bacteria aggregate to each other or adhere to a living (biotic) or non-living (abiotic) surface. The biofilm grows by cell division and by recruiting surrounding cells. In the disaggregation stage, bacteria leave the biofilm as aggregates or individual cells.

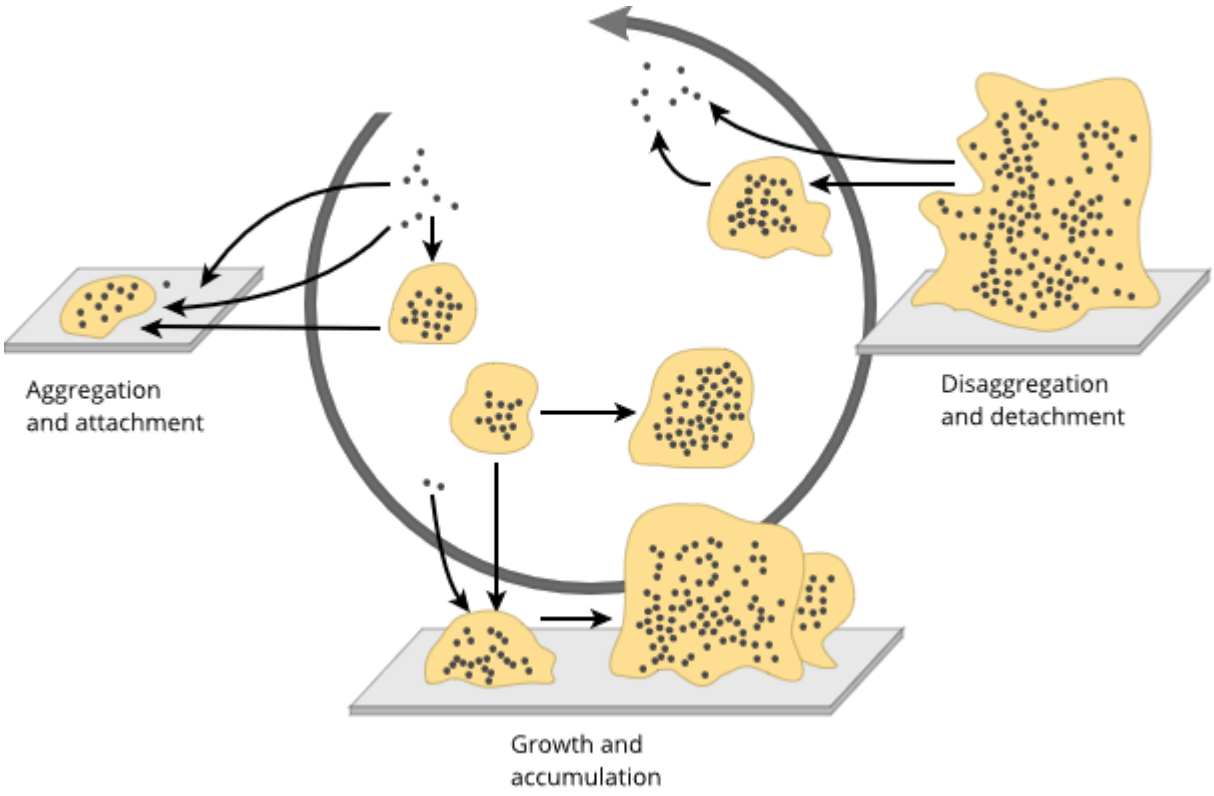

Figure 3 *- Biofilm formation model including the three major steps: aggregation and attachment, growth and accumulation, and disaggregation and detachment. Single bacterial cells are depicted as dots, and yellow shapes illustrate biofilms. Bacteria can enter the model at any stage. Surface-attached biofilms are represented in the outer ring, while the non-surface-attached are indicated by the inner ring, allowing potential interchange between them. Adapted with permission from [13].*

The most studied staphylococcal biofilms are the ones produced by *S. aureus* and *S. epidermidis*, but biofilm formation is also a common phenotype among *S. haemolyticus* [92, 93]. However, the structure and composition of biofilms are unequal between the species. Proteins and extracellular DNA are important components of the *S. haemolyticus* biofilm matrix [23, 92-95]. This contrasts with *S. epidermidis* where the *ica* operon and production of polysaccharide intercellular adhesin (PIA) plays a major role [96]. However, even though the *ica* operon is not commonly reported in *S. haemolyticus*, there are examples of strains that carry it [44, 97].

Within a mature biofilm, cells in the outer part are more metabolically active than those in the inner part. This is due to the reduced diffusion of oxygen and nutrients in the inner layer of the biofilm, creating an environment where cells may enter a dormant or persister state [65]. Reduced metabolic activity within the biofilm leads to increased tolerance to antibiotic treatment compared to planktonic cells, as antibiotics often target actively growing cells. For instance, a teicoplanin-susceptible *S. haemolyticus* strain from a cancer patient with a persistent

infection was resistant to teicoplanin treatment due to biofilm formation [98]. While some antibiotics are effective at the minimum inhibitory concentration (MIC), others need a 100 to 1,000 times concentration to eradicate a biofilm [99]. In a study by Ceri et al., they tested several antibiotics against planktonic cells and biofilms of *S. aureus* [99]. As an example, MIC for gentamicin was 0.5 µg/ml against planktonic cells and 2 µg/ml against biofilm. For oxacillin the MIC was 0.12 µg/ml against planktonic cells and >1024 µg/ml against biofilms [99]. Combinations of two or more antibiotics may eradicate biofilms more effectively than use of single antibiotics [100]. In a study by Szczuka, they found that combination treatment with tigecycline/rifampicin was significantly more effective than daptomycin/rifampicin against *S. haemolyticus* biofilms [93]. The physical barrier of the biofilm matrix also protects the bacterial cells from the host immune system. A primary strategy for managing biofilm infections is to remove the source of infection, such as a medical device [100].

Regulation of biofilm formation involves multiple genetic and environmental factors. Quorum sensing is a communication system that coordinates bacterial cells growth based on cell density, and it plays a pivotal role in the regulation of biofilm formation [34, 94, 101]. In addition, environmental conditions, such as temperature, pH, nutrient availability, and stress conditions can influence the expression of biofilm-associated genes [102, 103]. This allows the bacteria to adapt to changing environments and persist in hostile conditions.

Biofilm production is more prevalent among clinical *S. haemolyticus* isolates (67%) than the commensal ones (35%) [23]. Only strong biofilm formers were found when investigating 34 *S. haemolyticus* isolates from intensive care unit patients with catheter related blood stream infection or colonized central venous catheters [104]. Mucoid phenotypes can produce more biofilm and be more resistant to certain antibiotics than non-mucoid isolates [105-107].

Several proteins are implicated in biofilm formation in staphylococci [31]. Cell wall-anchored proteins mediating adhesion include the serine-rich repeat containing proteins (Sdr and SraP) [31, 79, 83, 108]. Non-anchored surface associated proteins like the extracellular matrix binding protein (Embp) and autolysin (Atl) also contribute to adhesion and biofilm accumulation [31, 79, 109-111]. Autolysin was present in 97% of clinical *S. haemolyticus* strains in a Brazilian study [103]. In our own research, autolysin had significantly increased abundance after surface shaving of *S. haemolyticus* that was incubated with human keratinocytes, compared to those with no contact with human cells [79].

1.2.3 Immune evasion and influence

Our immune system is the body's defence against pathogens, and consists of specialised cells, organs and soluble proteins that can disarm the invaders. The immune system can be divided into the innate and the adaptive immune defence. The innate immune system offers immediate, nonspecific defences (e.g. skin, phagocytic cells), whereas the adaptive system develops over time to provide specific responses (e.g. antibodies, T-cells) tailored to particular pathogens [112]. The complement system and the cytokine network are key components of the innate immune system's inflammatory response [112]. The complement system activates through classical, lectin, or alternative pathways [113]. Opsonization of bacteria by complement proteins enhances the binding and uptake by phagocytic immune cells, facilitating more efficient recognition and elimination of pathogens. However, phagocytes can also directly recognize pathogen-associated molecular patterns (PAMPs) found on bacteria. The direct recognition is mediated through pattern recognition receptors (PRRs) on the surface of phagocytes. Cytokines, such as interleukin (IL)-6, IL-8, and tumour necrosis factor (TNF)-α are small proteins released by immune cells. They serve as signalling molecules to regulate responses to infection, inflammation, and trauma, directing initiation and coordination of immune reactions [112]. The host recognize bacteria by surface receptors and intracellular pattern recognition receptors [114]. The initial defence against staphylococci includes activation of the complement system and killing of the bacteria by specialized immune cells, such as macrophages and polymorphonuclear leukocytes [113-115].

NAS use several strategies to protect themselves from the host immune system, where biofilm formation is the most important strategy [65]. The biofilm offers mechanical protection against phagocytosis, protects against antimicrobial peptides, and shields pathogen-associated molecular patterns on the bacterial surface [113]. Staphylococci can alter their phenotype in response to harsh environmental conditions, including exposure to antibiotics and the defences of the host immune system. These phenotypic shifts can lead to biofilm formation, persister cells and small colony variants, all of which contribute to bacterial survival [116, 117]. Cells in biofilms are more protected from the host immune system. Persister cells are dormant cells that may arise in the inner layer of a biofilm due to the reduced diffusion of oxygen and nutrients [65]. Slow-growing subpopulations of bacteria that have adapted to an alteration in environmental conditions are called small colony variants. They may reside intracellularly and are thus better protected against antibiotics and the host immune response [116]. Small colony variants can be persisters, but not all persisters are small colony variants [118]. The small colony

variant phenotype can cause chronic and relapsing infections, such as chronic prosthetic joint infections [119]. Small colony variants might change their phenotype to non-small colony variants under laboratory conditions [117]. Another phenotype that offers protection against the host immune system is the production of a polysaccharide capsule. Capsular operons are described in *S. haemolyticus* [23, 68, 84] and shield against uptake and killing by human neutrophils [120]. *S. haemolyticus* are also able to internalize host cells, and thereby protect themselves from the host immune system [121].

S. haemolyticus influences the immune system by producing toxins and enzymes [122]. *S. haemolyticus* produces PSMs, which are cytolytic peptide toxins that strongly promote pro-inflammatory activity, neutrophil chemotaxis, and cytokine release [61]. PSMs can kill neutrophils, playing a crucial role in staphylococcal immune evasion [123]. PSMs can trigger the overwhelming immune response typically for sepsis [61]. Studies have shown that most clinical *S. haemolyticus* strains carry at least one enterotoxin gene. One study analysed ten strains from diabetic foot ulcers [121], while another examined 84 blood culture isolates from hospitalized patients [124]. Enterotoxins function as superantigens, triggering an exaggerated immune response [125]. Secreted enterotoxins can kill host cells and cause disease by inducing cell death, such as apoptosis and necrosis [126]. *S. haemolyticus* can also produce cytotoxins [124], which can cause damage to host cells, including immune cells [122].

1.2.4 Antimicrobial use and resistance

Antimicrobials can have narrow or broad effect, and their mode of action is diverse [127, 128] (**table 1**). However, antimicrobial resistance can make the treatment ineffective. NAS are often associated with infections related to medical devices, and the primary strategy for managing infections is always to remove the source of infection. If NAS infections associated with intravascular catheters needs antibiotic therapy, The Norwegian Directorate of Health recommends standard treatment with cloxacillin and gentamicin [129]. In case of bacteraemia with NAS, vancomycin can be used if the strain is resistant to methicillin [129]. The choice of antibiotics for NAS often involves an evaluation of the antimicrobial susceptibility profiles and pharmacokinetics, with particular attention to toxicity and availability at the site of infection [130]. In Norway, cloxacillin is commonly used for methicillin-susceptible cases, but for more severe infections, treatment with vancomycin, daptomycin, rifampicin (in combination), ciprofloxacin (in combination), or linezolid (limited duration) may be applicable [130].

Linezolid is one of the final oral medication options for treating infections caused by staphylococci that are resistant to first-line antibiotics [131].

Table 1 *- Examples of antibiotic classes, generic names and mode of action on bacteria [128, 132, 133] with examples of S. haemolyticus resistance.*

ANTIBIOTIC CLASS (SUBCLASS)	GENERIC NAME	RESISTANCE	MODE OF ACTION
β-lactam (penicillin)	Methicillin	[106]	Cell wall synthesis
β-lactam (penicillin)	Oxacillin	[22, 40, 52, 134]	
β-lactam (penicillin)	Cloxacillin	[104]	
β-lactam (cephalosporin)	Cefoxitin	[62, 107, 134, 135]	
β-lactam (carbapenem)	Meropenem	*	
Glycopeptide	Vancomycin	[52]	
Lipopeptides	Daptomycin	[52]	Cell membrane function
Aminoglycoside	Gentamicin	[21, 22, 40, 52, 62, 107, 134, 135]	Protein synthesis (30S)
Tetracycline	Tetracycline	[21, 22, 40, 52, 62, 134]	
Tetracycline	Tigecycline	[52, 62]	
Oxazolidinone	Linezolid	[52, 62, 106, 107]	Protein synthesis (50S)
Macrolide	Erythromycin	[21, 22, 40, 52, 62, 104, 106, 107, 134, 135]	
Lincosamide	Clindamycin	[40, 52, 62, 104, 106, 107, 134, 135]	
Fluoroquinolone	Ciprofloxacin	[21, 22, 40, 62, 104, 134]	DNA synthesis
Rifamycin	Rifampicin	[40, 52, 62, 134]	RNA synthesis
Miscellaneous	Trimethoprim	[22, 40, 52, 134, 135]	Folate synthesis

**Susceptibility of staphylococci to carbapenems is inferred from the cefoxitin susceptibility, according to guidelines [132].*

S. haemolyticus is known for its high level of antibiotic resistance [22, 23, 62, 65, 135, 136]. The habitat influences the resistance, and clinical *S. haemolyticus* are more resistant than commensals [23, 62]. A study by Pain et al. compared 169 *S. haemolyticus* isolates, where most

of the clinical isolates were collected from human blood (Europe (majority), Japan, USA), and the commensal isolates originated from human skin (Norway) [21-23]. Eighty-eight % of the clinical and 11% of the commensal isolates were classified phenotypically as multi drug resistant [21-23]. Resistance genes enriched in clinical isolates were *aacA/aphD* (aminoglycoside resistance), *mecA* and *blaZ* (penicillin resistance). Macrolide resistance was common in both clinical and commensal isolates, but the genes causing the resistance varied [23]. In a similar study from Malaysia on 148 *S. haemolyticus* isolates, 54.1% of the clinical and 20.0% of the commensal isolates were multi-drug resistant, and clinical isolates were more resistant than commensals [62].

Many antibiotic resistance genes are located on mobile genetic elements, such as plasmids [137]. These can contribute to spread of resistance genes between staphylococci strains and species by horizontal gene transfer. *S. haemolyticus* and other NAS can thereby function as a reservoir of antimicrobial resistance genes to other bacteria such as *S. aureus* [30, 63, 64]. In a study by Fišarová et al., they detected identical plasmids, carrying resistance genes, in *S. haemolyticus*, *S. aureus* and *Staphylococcus petrasii* strains [64]. Smith and Andam investigated 1,876 NAS genomes delineated into 55 species and found that *S. haemolyticus* was in the group with the highest frequencies of receipt and donation of recombined DNA [30]. Genes for antimicrobial resistance were among the most common among recombined genes. Specific NAS species, such as *S. haemolyticus*, serve as hubs for gene exchange and are major reservoir of genetic variation within the genus [30].

1.3 Bacteriocins

One of the strategies to defeat antimicrobial resistance is to search for new antimicrobial substances. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria to inhibit growth of often closely related bacterial species. They act as a defence mechanism in microbial warfare, where neighbouring bacteria compete for the same resources. Bacteriocin-producing bacteria can be found on various locations, such as the skin [138, 139] and gastrointestinal tract [140, 141]. Bacteriocins can be narrow spectrum, targeting specific strains, or broad spectrum, affecting a wider range of bacteria [142, 143]. They are often effective at low concentrations and are stable at high temperatures and extreme pH levels [142]. The mechanism of action can be very specific and different from antibiotics, making them a potentially valuable alternative in the fight against antibiotic-resistant bacteria [142]. The

modes of action can broadly be categorized into two groups. The first group targets the cell envelope, such as forming pores in the cytoplasmic membrane. The second group acts within the cell, affecting gene expression, protein production, or targeting specific transporters [142, 144-146]. Combining different bacteriocins or pairing them with bioactive molecules like antibiotics can enhance their effectiveness [147-151].

Bacteriocin classification is based on the presence or absence of post-translational modifications. Class I bacteriocins are post-translationally modified peptides, while class II are unmodified peptides [142]. Lantibiotics are lanthipeptides with antimicrobial properties and belong to Class I bacteriocins [142, 152]. Lantibiotics are ribosomally synthesized as precursor peptides, comprising an N-terminal leader peptide and a C-terminal core peptide [153, 154]. The core peptide undergoes post-translational modifications, while the leader peptide facilitates these modifications and keeps the peptide inactive until secretion. These modifications involve the formation of thioether cross-links between cysteine and dehydrated serine or threonine, resulting in the unusual amino acids lanthionine and methyllanthionine. After these modifications, the leader peptide is cleaved off, releasing the active lanthipeptide into the extracellular space [153, 154]. Many lantibiotics target lipid II, a precursor in peptidoglycan synthesis, thereby inhibiting peptidoglycan synthesis and facilitating pore formation [142, 154]. Some lantibiotics are two-peptide bacteriocins, where two peptide components (α and β) act synergistically to get maximal antimicrobial activity [155, 156]. Bacteria express specific genes that provide immunity to their own bacteriocin. These include genes for an ABC transporter that export the bacteriocin out of the cell, and for immunity proteins that provide internal protection [157, 158].

A well described lantibiotic is nisin from *Lactococcus lactis*. It is used as a food preservative due to its effectiveness against a range of foodborne pathogens, its safety and low toxicity [159]. Research indicates that nisin's protective effect in bacterial infections extends beyond direct killing of bacteria and additionally involves modulation of the host immune response to effectively clear the infection [160]. Nisin has been shown to modulate the cytokine responses, affect immune cells, and have an anti-inflammatory effect in the infected organism [160]. Nisin kill target cells by forming pores in the cytoplasmic membrane, or it can prevent cell wall synthesis [161]. Nisin has been used in the food industry since the 1960s [159], and despite the widespread use, hardly any acquired resistance has been described [153, 162]. Nisin has also

been explored in clinical trials as an alternative to antibiotics, such as in a study by Fernández et al. demonstrating its efficiency in treating staphylococcal mastitis [163].

Several staphylococci strains are known to produce bacteriocins [65, 146, 164, 165]. Our own research identified a bacteriocin in *S. haemolyticus* that is effective against a broad range of Gram-positive species, including vancomycin-resistant *Enterococcus faecium* (VRE) and methicillin-resistant *S. aureus* (MRSA). It also eradicated biofilms of *S. haemolyticus*, *S. epidermidis*, MRSA, and VRE [139]. MRSA and VRE are classified as global high priority pathogens by WHO where we urgently need new antimicrobial treatment options [166].

Some bacteriocins are under investigation for therapeutic applications [146, 167, 168]. However, despite their promising antimicrobial properties, the use of bacteriocins in the veterinary and medical sectors remains limited [142]. This is partly due to insufficient scientific data on the safety, toxicity, and immunomodulatory effect of bacteriocins on humans and animals [142, 146, 169]. Bacteriocins are generally considered to be nontoxic for mammalian cells, but some are toxic, such as the enterococcal cytolysin [170]. Evaluating toxicity is therefore important before initiating clinical trials. In a review by Benítez-Chao et al., they found that half of the studies testing bacteriocins on mice lacked toxicity assays [169]. To be used in a clinical setting, the safety, stability, and toxicity of bacteriocins and the resistance mechanisms towards them must be investigated.

1.4 Introduction summary

S. haemolyticus can be a harmless skin commensal or an opportunistic pathogen and are often very resistant to antibiotics. The interaction this species has with host cells or other bacteria are influenced by surface proteins, biofilm formation and bacteriocin production – aspects which are not yet well-understood. Understanding how *S. haemolyticus* interacts with its host and the environment is key to finding new ways to treat the infections it causes. This is the focus of this thesis.

2 Aims

The overall aim of this PhD project was to deepen our knowledge of virulence factors and bacteriocin production in *S. haemolyticus*. Enhanced understanding can lead to more effective strategies for preventing and treating infections caused by this bacterium.

Paper I – Surface proteins

Our objective was to investigate the adhesive and biofilm forming capacities of clinical and commensal *S. haemolyticus* strains. We aimed to use a novel method to identify *S. haemolyticus* surface proteins expressed during human host colonisation.

Paper II – A surface protein in detail

Our aim was to select a specific surface protein of *S. haemolyticus* and create a mutant strain lacking this protein. We then wanted to investigate changes in adhesion, biofilm formation, and immune activating effect in blood in mutant compared to wild type strain.

Paper III – Bacteriocins

We aimed to examine the bacteriocin production in *S. haemolyticus* and investigate if bacteriocins inhibit clinically relevant strains.

3 Materials and methods

This work is basic research on *S. haemolyticus*. The purpose of basic research is to expand our knowledge by exploring the fundamental principles and mechanisms of a phenomenon. It lays the groundwork for further scientific exploration and can eventually lead to applied research and technological innovations. Research on *S. haemolyticus* is scarce, and understanding the interactions between the bacterium, the host, and the environment is essential for developing novel treatments for the infections it causes.

Research in Norway is regulated in the Research Ethics Act [171] and supplemented by the European guidelines for research integrity [172]. Research integrity encompasses the reliability of research quality, honesty and transparency, respect, and accountability for the research [172]. Our work strives to follow these principles to produce credible and reproducible knowledge. Nevertheless, it is important to recognize that all methods have limitations, and these should be considered when interpreting the data.

This section is based on methodologies from two published studies [79, 139] and one manuscript in preparation. I provide a general overview of methods used in the manuscripts, and content parallels with these sources are anticipated. Detailed descriptions can be found in the attached papers. To enhance the reliability and interpretability of our results, we have used at least three biological replicates, several technical replicates, and controls wherever feasible. This approach ensures that our findings are robust, reproducible, and reflect a true biological variation rather than experimental error or artifact. Some assays are sensitive to external factors. Examples for how these can be minimized include using the same equipment and batches of reagents, run all samples on the same day or over a short period of time, and that the same person performs the experiments.

3.1 Study design

3.1.1 Paper I – Surface proteins

The study investigated the interaction between human cells and *S. haemolyticus*. We examined the adhesion and biofilm formation of ten clinical and ten commensal strains. Adhesion tests were conducted on plain plastic, plastic coated with host proteins fibrinogen and collagen, and human skin cells (keratinocytes). For in-depth analysis of the proteins expressed by *S. haemolyticus* during keratinocyte colonization, we selected a clinical strain for surface protein shaving after adhering to keratinocytes, using a lipid-based protein immobilization flow cell. The same strain treated similarly but without contact with human cells was included for comparison. The proteins were labelled with tandem mass tags and quantified via liquid chromatography to identify the expressed surface proteins present under each condition.

3.1.2 Paper II – A surface protein in detail

The study focused on one serine-rich repeat surface protein of *S. haemolyticus*. We created mutants without the protein (*ΔsraP*) and one of its accessory secretion system proteins (*ΔsecA2*), and evaluated the impact of the knockouts on adhesion, biofilm formation, blood survival and cytokine response. Adhesion tests were conducted on plastic coated with host proteins fibrinogen and fibronectin, and on human keratinocytes and lung cells.

3.1.3 Paper III – Bacteriocins

The study investigated whether *S. haemolyticus* produces bacteriocins with inhibitory effects on other bacteria. We analysed 174 clinical and commensal *S. haemolyticus* for antibacterial activity. Using whole-genome sequencing, purification, mass spectrometry and structure prediction, we discovered a novel bacteriocin in a strain from a healthy carrier. We also heterologously expressed the gene cluster in a different host, a *S. aureus* strain. We evaluated bacteriocin stability across various temperatures, pH levels, and protease exposure. Its antibacterial efficacy was tested against a wide range of species. The impact on staphylococci and enterococci biofilms was examined by confocal microscopy. We explored the bacteriocin's mode of action through a pore formation assay, scanning electron microscopy, growth curves, and a membrane integrity test.

3.2 Bacterial collection

The *S. haemolyticus* collection used in this work included 123 human clinical isolates, 46 human commensals, 4 animal isolates, and one type strain (CCUG 7323T) [21-23, 59].

In **paper I**, we performed adhesion and biofilm assays with ten clinical and ten commensal isolates. The clinical isolates originated from blood (8), wound (1), and one was of unknown origin. The commensal isolates originated from groin (5), armpit (2), hamstring (1), nose (1), and unknown (1). The bacterial surface protein shaving in **paper I** and creation of surface protein mutants in **paper II** was performed with a clinical wound strain isolated in Switzerland in 2004 (**table 2**). In **paper III** we screened all the 174 *S. haemolyticus* isolates in our collection for bacteriocin inhibitory activity, and the bacteriocin producing strain originated from the groin of an asymptomatic carrier, collected in Tromsø in 2013 (**table 2**).

***Table 2** – Overview of the main S. haemolyticus strains used in the thesis.*

Lab-ID	ENA ID*	Source	Paper I	Paper II	Paper III
53-38	ERS066380	Wound	x	x	
57-27	ERS3370787	Groin of asymptomatic carrier			x

** ENA: European Nucleotide Archive*

3.3 Host-microbe interaction

Staphylococcal surface proteins facilitate interactions with host receptors, other bacteria, macromolecules (such as fibrinogen, collagen, or fibronectin), and abiotic surfaces including medical devices. Surface proteins are important for virulence and biofilm formation [72, 74, 75]. In **paper I** and **II**, we have investigated the details of surface proteins, adhesion, biofilm formation, and survival of *S. haemolyticus*.

3.3.1 Bacterial protein surface shaving

In **paper I**, we wanted to investigate the expression of surface proteins in a clinical strain of *S. haemolyticus* during colonization of human keratinocytes (HaCaT). The chosen strain had strong adhesion and biofilm-formation capabilities, which are important virulence factors in the species. We developed a novel method for bacterial surface shaving, and **figure 4** summarize the workflow [79]. *S. haemolyticus* was co-incubated with confluent growing HaCaT cells in

tissue culture plates for 1 hour, and a control sample was grown under similar conditions, but without human cells. Bacteria adhering to HaCaT cells were collected through mechanical scraping, avoiding trypsin-treatment which could alter the proteins on the bacterial surface. Bacteria were labelled with a fluorescent dye and separated from human cells using a fluorescence-activated cell sorting system (FACS) based on size and fluorescence. After FACS, bacterial samples were concentrated and loaded into a lipid-based flow cell, where surface expressed proteins were digested (shaved) with trypsin. The lipid-based protein immobilization technology was developed by our collaborators in Nanoxis®, where bacteria attach to gold coated channels in the flow cell. Proteomic analysis was performed at the Proteomics Core Facility at Gothenburg University. Peptides were labelled with tandem mass tags (TMT) to allow quantification and analysed by liquid chromatography mass spectrometry (LC-MS/MS). LPxTG motifs were predicted using a manual sequence search. Subcellular localizations were determined using PSORTb, CELLO, and LocateP. We defined surface proteins as proteins predicted from cytoplasmic membrane, cell wall or extracellular origin. Functional annotation of proteins was done with EggNOG, PHMMER, and protein BLAST. Moonlighting proteins were identified using the MoonProt database and published literature.

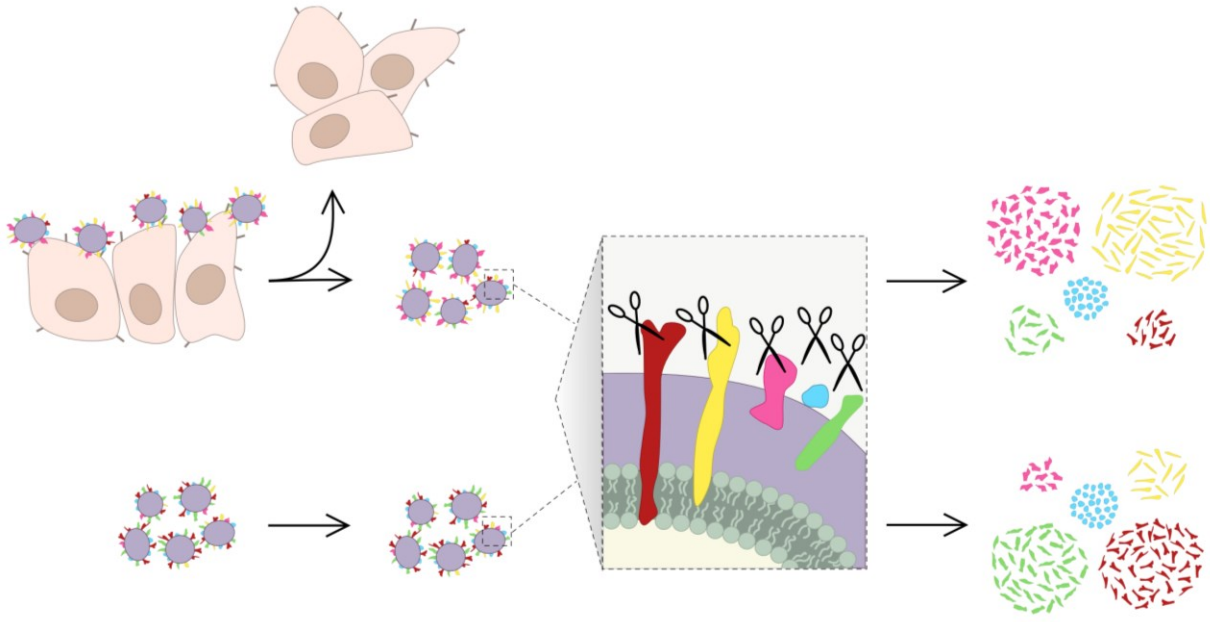

Figure 4 *- Bacterial surface protein shaving, graphical abstract. Comparison of S. haemolyticus surface protein expression after HaCaT colonization (top) and the control group (bottom). Bacterial surface proteins (multi-coloured) are degraded by the trypsin protease (scissors). Figure from [79].*

3.3.2 Creating mutants

In **Paper II**, we wanted to create *S. haemolyticus* mutants without the SraP surface protein and its accessory secretion system and look at the impact of knockouts. SraP is a part of the serine-rich repeat proteins in Gram-positive bacteria that facilitate attachment to host and bacterial surfaces [85]. Briefly, we used the allelic exchange method described in Monk et al. [173, 174], which had been adapted for use in *S. haemolyticus* [175]. Approximately 500 bp upstream and downstream of the genes of interest were amplified by polymerase chain reaction (PCR) and used to create a template for allelic exchange and inserted into the plasmid pIMAY-Z and electroporated into competent 53-38 cells. The temperature-sensitive replicon and *Lac-Z* gene (in combination with X-gal in the media) provided means of selecting colonies which successfully transformed, integrated, and then excised the plasmid, using the allelic exchange template to eliminate the gene of interest from the chromosome (**figure 5 and 6**). Elimination of the *sraP/secA2* genes was determined first by PCR and later by whole genome sequencing. We performed a growth curve analysis of *S. haemolyticus* wild type and mutants measuring optical density over 24 hours to assess the impact of the mutations on growth.

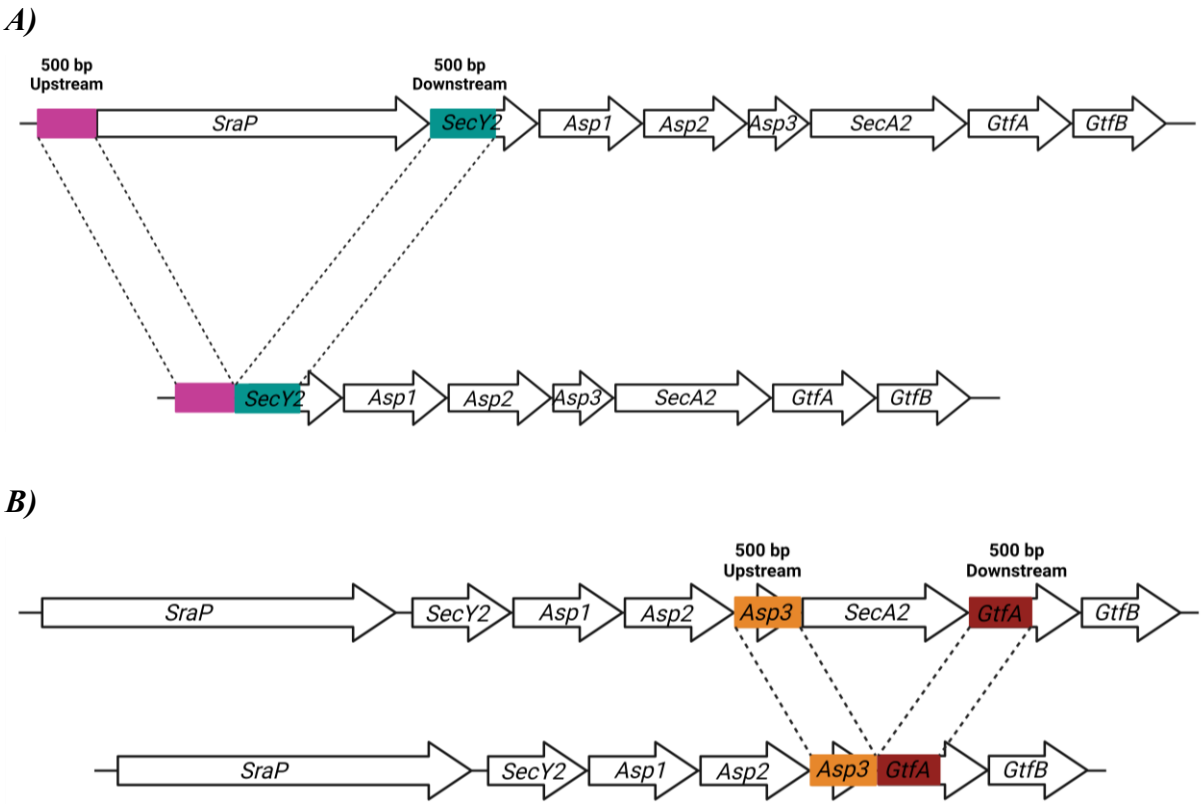

***Figure 5** – Knockouts of **(A)** ΔsraP and **(B)** ΔsecA2 genes in S. haemolyticus wild type. Figure by H. Venter, reproduced with permission.*

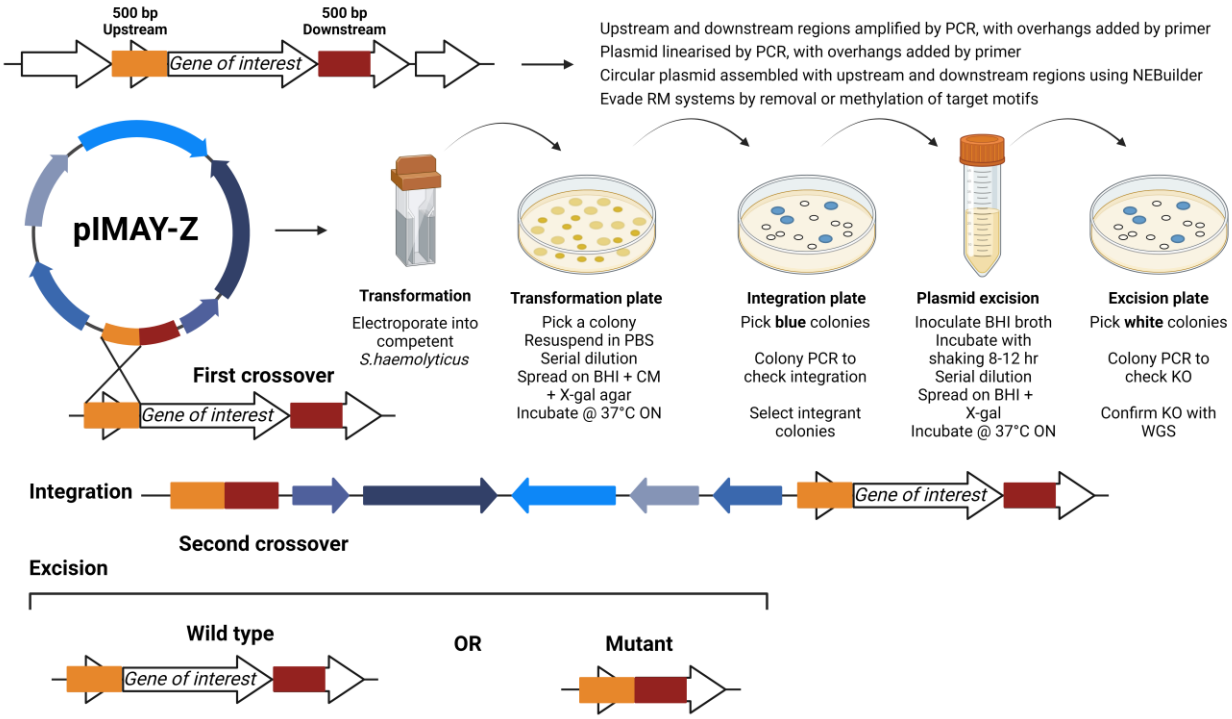

Figure 6 *– Creating mutants, graphical abstract. Figure by H. Venter, reproduced with permission.*

3.3.3 Adhesion to plastic and host proteins

We explored the adhesive properties of *S. haemolyticus* to various substrates using a microtiter plate-based assay [176]. After one hour of incubation, we determined the biomass of adherent bacteria to the substrates by staining with crystal violet before absorbance measurement. In **paper I**, we examined adhesion of ten clinical and ten commensal strains to plastic or plastic coated with fibrinogen and collagen. In **paper II**, we compared the ability of SraP wild type and mutants (*ΔsraP/ΔsecA2*) to adhere to fibrinogen and fibronectin. Collagen, fibronectin, and fibrinogen are human extracellular matrix proteins that provide structural support and mediate cellular processes, but they can also serve as binding sites for bacterial pathogens [177-179].

3.3.4 Biofilm formation

We performed a semi-quantitative biofilm formation assay on *S. haemolyticus* [92, 180]. We established biofilm in flat-bottom microtiter plates, and the biomass of bacteria was determined by staining with crystal violet before absorbance measurement. In **paper I**, we examined biofilm formation of ten clinical and ten commensal strains. In **paper II**, we compared the biofilm forming ability of SraP wild type and mutants (*ΔsraP/ΔsecA2*). Tryptic soy broth (TSB) with 1% glucose was used as growth medium in both assays. In **paper II**, we additionally used spent human cell medium.

3.3.5 Adhesion to and survival in human cells

We wanted to test how *S. haemolyticus* adhered to and survived in human cells. We included human keratinocytes due to *S. haemolyticus'* presence in the skin microbiota [21, 26], and lung cells to evaluate the bacterium's behaviour across different cell types and align with similar studies. We used a modified protocol based on Edwards and Massey [181]. We cultured human cells to confluency in tissue culture plates before bacterial addition. We investigated the adhesion (40-60 minutes) of *S. haemolyticus* to human keratinocytes (HaCaT) (**paper I and II**) and lung epithelial cells (A549) (**paper II**). We centrifuged the plates after inoculating bacteria to human cells to allow a more rapid contact between them. In **paper II** we also investigated survival (20 hours) to human keratinocytes and lung cells. Extracellular bacteria were killed by antibiotics. The results in both adhesion and survival assays were determined by counting colony forming units (CFU). In **paper I** we examined ten clinical and ten commensal strains, and in **paper II** we compared SraP wild type and mutants (*ΔsraP/ΔsecA2*).

3.3.6 Immunology

In **Paper II**, we wanted to examine how *S. haemolyticus* SraP wild type and *ΔsraP/ΔsecA2* mutants' survived in blood and affected the human cytokine response.

For the blood survival experiment, we used a previously described method [182]. We mixed bacteria with freshly drawn blood from healthy adult donors and incubated for three hours and lysed the blood cells. We evaluated bacterial survival rates by comparing CFU after the experiments with the CFU in the initial inoculum.

With a few modifications, we used an *ex vivo* human whole-blood model as previously described [183]. We collected blood from children undergoing chemotherapy with a reduced immune defence and from healthy adults with intact immune systems to examine the differences. We used lepirudin as an anticoagulant thrombin-inhibitor as it does not interfere with the complement system or the inflammatory network [184]. We mixed blood with bacteria and incubated for four hours and included a control without bacteria. The complement activation was stopped by placing the samples on ice and they were stored at −70 °C. We collected plasma and measured pro-inflammatory cytokines (TNF, IL-6, and IL-8) using a multiplex cytokine assay.

3.4 Bacteriocin assays

Bacteriocins, produced by bacteria to suppress rival species, can have a mode of action distinct from conventional antibiotics and may contribute to combating antimicrobial resistance. In **paper III**, we identified and purified a bacteriocin from *S. haemolyticus*, investigated its mode of action, and examined its range of antibacterial activity.

3.4.1 Inhibition of growth

We screened 174 clinical and commensal *S. haemolyticus* isolates for bacteriocin activity by applying overnight cultures to lawns of clinical *S. haemolyticus* and *S. aureus*. *L. lactis* was also included due to its susceptibility to various bacteriocins. After overnight incubation, the presence of clear zones indicated growth inhibition. To investigate bacteriocin stability, we exposed concentrated cell-free supernatants to various temperatures (4-121°C), pH (2.1-11.9), and protease treatment (trypsin) prior to antimicrobial assays. We also submitted the *S. haemolyticus* genomes to the BAGEL4 webserver for identification of bacteriocin genes.

To confirm the function of the bacteriocin genes, we expressed the bacteriocin cluster in a different species (heterologous expression), that naturally lacks these specific genes. We cloned the bacteriocin genes into an inducible expression vector and transformed it into a *S. aureus* strain by electroporation. The plasmid vector allows anhydrous tetracycline-inducible expression of cloned genes. To activate bacteriocin gene expression, we added anhydrous tetracycline to the overnight culture medium of transformed *S. aureus.* The bacteriocin activity

was then assessed by applying the cell-free supernatant from these cultures to a lawn of *L. lactis* as described in the previous section.

To determine the bacteriocin spectrum of activity, we conducted spot-on-lawn and planktonic growth inhibition assays against WHO priority pathogens and a broad range of Gram-positive species. We used a similar method as Holo [185] for the spot-on-lawn assay. It involved embedding of target bacteria in soft agar, plating it out as a lawn on brain heart infusion (BHI) agar plates, and applying the purified bacteriocin on the lawn before overnight incubation. Growth inhibition appeared as clear zones. Planktonic growth inhibition was evaluated in 96-well plates following the colony suspension and broth microdilution for antimicrobial peptides methods in the Wiegand protocol [186].

We investigated the efficacy of the bacteriocin to disrupt biofilms of bacteria commonly implicated in infections associated with medical devices. Using confocal microscopy, we analysed the impact of the bacteriocin on *S. haemolyticus*, *S. epidermidis*, MRSA, and VRE biofilms. After establishing biofilms in four-well glass slides, we added bacteriocin or negative control and incubated overnight. Cells were stained with live/dead staining before confocal microscopy.

We examined the bacteriostatic or bacteriolytic effects of the bacteriocin by monitoring the growth curves of *S. haemolyticus* and MRSA in presence of bacteriocin. We measured optical density every ten minutes over a 21-hour time-period and counted CFU at 0 and 21 hours.

3.4.2 Purification

We purified the *S. haemolyticus* bacteriocin to facilitate a more detailed examination of its properties [187]. We precipitated proteins with ammonium sulphate, followed by cationic exchange and reversed-phase chromatography with a 2-propanol gradient. Antimicrobial activity was determined using *L. lactis* in a 96-well plate assay and spectrophotometrically measurement of growth. We pooled active fractions from a total of four litres of culture and analysed them by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). To confirm if the molecules matched the lantibiotic found in the genome, we conducted a structure prediction for the modified peptides to calculate their expected mass.

3.4.3 Mode of action

We employed three techniques to elucidate the mode of action of the bacteriocin, namely: a propidium iodide pore formation assay, scanning electron microscopy (SEM), and a luminescence membrane integrity assay.

Propidium iodide is a fluorescent compound that exhibits increased fluorescence intensity when it intercalates with DNA. Intact bacterial cells are impermeable to the compound, but it will diffuse into cells with a damaged membrane, leading to an increase in fluorescence signal. We added *L. lactis* to each well of a black microtiter plate containing bacteriocin and propidium iodide. We used Nisin A and Micrococcin P1 to compare our bacteriocin with. Fluorescence was kinetically measured in a microplate reader every 10 minutes for 3 hours to assess membrane damage.

We performed SEM to visualize potential bacteriocin-induced morphological changes on bacterial cells. We treated bacterial cells with bacteriocins, and then fixed, dehydrated, and dried them before imaging. We used the same *L. lactis* strain as in the pore formation assay, but also performed SEM on MRSA, *S. haemolyticus*, *S. epidermidis*, and *Bacillus subtilis* to determine if the mode of action is species dependent. We used cultures with no bacteriocin added as controls.

We evaluated the membrane disruptive properties of the bacteriocin by using a previously described bioluminescence assay [188]. The method uses a *B. subtilis* strain constitutively expressing luciferase, and that emits light if its membrane is permeabilized and D-luciferin substrate enters the cell. ATP is needed for light to be emitted. A decrease in luminescence indicates cell death due to ATP loss. We used chlorhexidine as a reference, which is known for its membrane disruptive properties. Antimicrobial compounds were combined with *B. subtilis* in black round-bottom 96-well plates, and luminescence was measured in a microplate reader for a duration of 0-4 minutes following the addition of the antimicrobial compound.

3.5 Ethical considerations

In **paper I** and **III**, we did not conduct any experiments on humans or animals that required consent to participate or an approval from an ethical committee.

In **paper II**, the regional committee for medical research ethics approved the collection of blood from neutropenic children (REK 566236) and healthy adults (REK 741976). Informed consents were obtained, and the participants could withdraw from the study at any time.

3.6 Funding

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3.7 Language tools

To enhance the language of this summary, I have utilized various resources, including dictionaries (such as Ordnett, Google Translate, Merriam-Webster), input from colleagues proficient in academic English, and AI tools (such as Chat UiT GPT-4, Microsoft Bing Copilot). AI tools were employed solely for refining the language, not for text generation.

4 Summary of main results

Paper I: Identification of surface proteins in a clinical *Staphylococcus haemolyticus* isolate by bacterial surface shaving

We aimed to better understand the interaction between human cells and matrix components and *S. haemolyticus*. We investigated adhesion and biofilm formation in clinical and commensal strains. All formed biofilm, and clinical strains showed higher biofilm production. We used bacterial surface shaving on one clinical strain to identify expressed surface proteins after bacterial adhesion to human skin cells. Our research has pinpointed surface proteins previously noted only in other staphylococci. We have also detected hypothetical surface proteins with unknown function.

- We investigated adhesion and biofilm formation of ten commensal and ten clinical *S. haemolyticus* strains. To examine adhesion, we used uncoated plastic or plastic coated with host proteins fibronectin or collagen. We also tested adhesion to human keratinocytes (HaCaT cells).
- All strains adhered to uncoated plastic, but the binding was generally low. There was no significant difference between clinical and commensal strains. There was low binding to fibronectin and collagen for all strains, but significantly higher for commensals.
- In three clinical and one commensal strain over 60% of the inoculum adhered to the keratinocytes. The other strains showed lower adhesion. In general, there was no statistically significant difference between the clinical and commensal strains regarding adhesion to keratinocytes.
- Biofilm formation was observed in all strains, but the formation was higher in clinical strains.
- We chose one strain with strong biofilm formation and high adhesion to HaCaT cells for bacterial surface protein shaving using a lipid-based protein immobilization flow cell. We labelled proteins with tandem mass tags prior to LC-MS/MS to perform relative protein quantification.
- We identified 325 bacterial proteins after contact with human keratinocytes, where 65 were classified as surface proteins, 11 as undefined proteins and 249 as cytoplasmic proteins.
- Of the identified surface proteins there were five LPxTG, one LPXSG and one LPXAG domain containing protein. Furthermore, we found three serine-aspartate-repeat (Sdr-like) proteins, the extracellular matrix binding protein (Embp), one Mannosylglucosyl-3-

phosphoglycerate phosphatase (SasH-like), and two uncharacterized surface proteins. Other well characterized surface proteins were the lytic transglycosylase immunodominant staphylococcal antigen A (IsaA), the Immunodominant staphylococcal antigen B (IsaB) and the elastin binding protein (EbpS).

- We examined if the protein abundance differed when *S. haemolyticus* colonized HaCaT cells compared to when they were grown in cell culture media supplemented with serum. The abundance of most proteins, including EbpS, IsaB and cytoplasmic proteins, remained consistent across both conditions compared. Only 19/325 proteins (5.8%) had a significant change in abundance following HaCaT colonization. The surface proteins lytic transglycosylase Staphylococcus epidermidis D protein (SceD) and autolysin Atl showed significantly increased abundance after HaCaT co-incubation. In addition, the Toll/interleukin-1 like (TIRs) domain had increased abundance. The staphylococcal secretory antigen (SsaA) was significantly decreased.
- Of the predicted cytoplasmic proteins, we found eleven with moonlighting function, both engaged intracellularly and with adhesive functions extracellularly.

Paper II: *Staphylococcus haemolyticus* SraP promotes binding to human cells

We made mutants of the surface protein SraP (*ΔsraP*) and its accessory secretion system (*ΔsecA2*) in *S. haemolyticus*. Compared to the wild type, the mutants showed lower adhesion to and survival in human skin cells and lung cells, as well as reduced survival in blood. There was no difference between wild type and mutants in forming biofilm in conventional growth medium, and we did not observe a strong immune response from pro-inflammatory cytokines.

- The SraP and the accessory secretion system in *S. haemolyticus* consists of the substrate gene (*sraP*), a protein translocase gene (*secY2*), three accessory secretory protein genes (*asp1-3*), a transport-associated ATPase gene (*secA2*) and two glycosyltransferase genes (*gftA* and *gftB*).
- The SraP protein sequences are conserved across different species and showed 57.46% sequence similarity with *S. aureus* USA300 and 82.86% with the *S. haemolyticus* JCSC1435 type strain.
- The adhesion to fibrinogen and fibronectin was low for both wild type and *ΔsraP*/*ΔsecA2* mutants with levels similar to the blank.
- Biofilm formation between wild type and mutant was not significantly different after 24 hours in TSB with 1% glucose. In spent HaCaT medium, the biofilm formation was slightly increased for *ΔsraP* after 24 hours compared to the wild type, but it was not evident after 48 hours. Biofilm growth was higher in TSB with 1% glucose compared to spent HaCaT medium.
- The *ΔsraP*/*ΔsecA2* mutants showed reduced adhesion to and survival in human keratinocytes (HaCaT) and lung epithelial cells (A549) compared to the wild type. The difference between wild type and mutants was highest in HaCaT cells (significant reduction). In HaCaT cells, the reduction in growth for mutants compared to the wild type was over 60% after adhesion and over 75% after the survival assay.
- In plasma from healthy adults and immunocompromised children in the non-neutropenic phase, the *ΔsraP*/*ΔsecA2* mutants showed higher levels of the pro-inflammatory cytokine IL-8 compared to the wild type, though the difference was not statistically significant.
- The wild type had significantly increased survival in blood compared to *ΔsraP*/*ΔsecA2* mutants.

Paper III: The novel bacteriocin romsacin from *Staphylococcus haemolyticus* inhibits Gram-positive WHO priority pathogens

In *S. haemolyticus*, we identified an operon/gene cluster encoding a novel bacteriocin. We studied the antimicrobial activity of the new bacteriocin, which we named romsacin after the Sami name of Tromsø (Romsa). Romsacin is produced by *S. haemolyticus* and is active against a broad range of Gram-positive bacteria. It works against planktonic cells and bacterial biofilms. Additional research is required to determine the therapeutic capabilities of romsacin, and to elucidate its structure and mode of action.

- We screened 174 *S. haemolyticus* clinical and commensal strains. One strain originating from the groin of a healthy carrier produced an antimicrobial substance.
- The strain contained a gene cluster with homology to a two-peptide lantibiotic bacteriocin, but the sequence identity to previously described lantibiotics was low.
- Whole-genome sequencing, purification, mass spectrometry, and structure prediction identified the antimicrobial as a new two-peptide lantibiotic. We named the new bacteriocin romsacin.
- We heterologously expressed the gene cluster in a different host, a *S. aureus* strain, and it gained the same antimicrobial properties as the romsacin producing *S. haemolyticus*.
- Romsacin was temperature stable (4°C–121°C), pH stable (2–12), and protease sensitive (trypsin), which are all characteristics of bacteriocins.
- Romsacin inhibited the growth of a broad range of Gram-positive species of animal and human origin, including the WHO priority pathogens VRE and MRSA. It was also effective against food-borne pathogens such as *Bacillus cereus* and *Listeria monocytogenes*.
- Romsacin did not inhibit Gram-negative strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*.
- Romsacin eradicated *S. haemolyticus*, *S. epidermidis*, MRSA, and VRE biofilms.
- Our results indicated that pore formation of the bacterial membrane was not the mode of action of romsacin in staphylococci and lactococci.
- Romsacin-treated *B. subtilis* showed rapid membrane leakage.
- When *S. haemolyticus* and *S. aureus* were treated with romsacin, their growth curves indicated a swift antimicrobial impact within two hours.

5 General discussion

S. haemolyticus is a multifaceted bacterium. It naturally resides in our microbiota [21, 31] and some strains can produce bacteriocins with antimicrobial effects [139]. However, it also acts as an opportunistic pathogen, making treatment challenging due to its biofilm production, immune evasion, and antimicrobial resistance [23, 31, 62-65]. Patients with weakened immune systems are particularly susceptible to infections [31, 36]. Advancements in medicine and an aging population are boosting the demand for medical implants and prosthetic devices [31]. Consequently, this could result in more frequent biofilm-related complications with the bacterium. The limited knowledge about *S. haemolyticus* underscores the need for further research. The objective of this thesis was to enhance our understanding of virulence factors and bacteriocin production in *S. haemolyticus*. A deeper insight into these areas could lead to improved strategies for preventing and treating infections caused by this bacterium. We have uncovered new insights into these aspects of *S. haemolyticus*, which will be discussed in detail below.

5.1 Host-microbe interaction

5.1.1 Surface proteins

Bacterial cell surface proteins are crucial for interacting with the host, facilitating adherence, biofilm formation, and immune evasion. [189]. Understanding these proteins can lead to new therapeutic strategies for treating infections. In **paper I**, we analysed the expression of surface proteins by shaving the surface of a clinical *S. haemolyticus* strain after contact with human keratinocytes. As *S. haemolyticus* is part of the skin microbiota [21, 31], this approach aims to create a more biologically relevant environment to identify expressed surface proteins than using conventional growth medium. As a control we used a sample that was treated similarly, but without contact with human cells.

Subcellular localization analysis and functional annotation following surface shaving identified 325 bacterial proteins after contact with human keratinocytes, where 65 were classified as surface proteins, 11 as undefined proteins and 249 as cytoplasmic proteins. Surface proteins in staphylococci have long repeat regions in their DNA sequences, which are challenging to assemble accurately with short sequence reads [190]. Despite these challenges, our study has

identified several interesting surface proteins previously only observed in other staphylococci. We have also detected hypothetical surface proteins of unknown function.

The surface proteins SceD and Atl were significantly more abundant after incubation with keratinocytes compared to the control without human cell contact. The lytic transglycosylase SceD is essential for normal growth and effective host-pathogen interaction [191, 192]. The bifunctional autolysin Atl is described in several staphylococcal species [65]. It is involved in cell wall turnover, cell division, lysis, and contributes to attachment to host matrix proteins, biofilm formation and DNA release [48, 65, 193, 194]. In *S. aureus*, Atl is essential for maintaining optimal levels of cell wall-anchored surface proteins. Disrupting autolysin function reduces the bacterium's affinity for host cell ligands [195]. Atl may also facilitate low-level internalization in host cells, which is necessary for development of therapy-resistant chronic infections [196].

Cell-wall anchored proteins have a sorting signal with an LPxTG motif [78]. *In silico* analysis of the clinical *S. haemolyticus* surface shaving isolate revealed 19 LPxTG containing genes, of which seven encoded adhesion proteins. After surface shaving, we identified five LPxTG, one LPxSG, and one LPxAG containing protein, including three MSCRAMM Sdr-like proteins. Sdr proteins, found in various staphylococci, can mediate binding to fibrinogen, collagen, keratin, keratinocytes, and epithelial cells [197-201]. In *S. aureus*, SdrD enhances survival and immune evasion in blood [182, 202]. A study on *S. aureus sdrD*, *sdrE*, and *sdrC* found that the *spa* type lacking *sdrD* and *sdrE* was least virulent in a mastitis mouse model. Another *spa* type with a deletion in the *sdrC* gene was the weakest biofilm producer [203]. SdrF in *S. epidermidis* may facilitate collagen binding and biofilm formation on breast implant surfaces [197], and it may adhere to human keratin to promote skin colonization [200].

A large proportion of the proteins identified after surface shaving were predicted as cytoplasmic. This occurrence is unavoidable with this technique, and may result from cell lysis, moonlighting proteins, or proteins released from membrane vesicles [204-207]. Moonlighting proteins are multifunctional. Among the predicted cytoplasmic proteins, we identified eleven that are known to have functions both intracellularly and with adhesive functions extracellularly. Membrane vesicles are released during bacterial growth, and it has been demonstrated that *S. haemolyticus* produces membrane vesicles mainly containing cytoplasmic proteins [208].

SraP facilitates binding of *S. aureus* to human platelets and lung epithelial cells, and its expression might be a virulence factor in endovascular infections [86, 91, 209]. The *sraP* homolog in *S. haemolyticus* is more prevalent in clinical than in commensal strains, and we wanted to investigate the protein more thoroughly. In **paper II**, we created SraP mutants, and we used the same strain as in the **paper I** surface shaving study. Although the *sraP* gene is present in the strain, we did not detect the protein after surface shaving, suggesting it was not expressed or not identified. One reason might be that the protein was not expressed under the experimental conditions. Before shaving, we incubated the bacteria with HaCaT cells, but we also did the same in the cell adhesion experiments in **paper II**. These experiments revealed that SraP mutants adhered significantly less to HaCaT cells than the wild type, indicating that SraP is expressed and contributed to cell adhesion. Another explanation might be that SraP may be a low-abundance protein, which could account for its lack of detection in the selected method. The trypsin treatment of the bacterial cell surface can also account for the lack of SraP detection. In a study by Ythier et al., the SraP protein was not detected in *S. aureus* after trypsin-shaving and LC-MS/MS analysis, despite the presence of its mRNA at the transcriptional level. However, it was detected in recombinant *L. lactis* using the same methods [210]. This suggests potential differences in trypsin accessibility between the species, possibly due to obstruction from other domains or glycosylation protecting the protein from proteolytic cleavage [211]. We used trypsin digestion in our surface shaving approach. Trypsin is a specific protease that cleaves peptide bonds at the carboxyl side of the amino acids lysine and arginine [204, 212]. In our strain, these residues are in the terminal region of the protein. This could have resulted in short peptides from the terminal regions and a long, uncleaved central region that the instrument may not have detected [204]. Using an additional, nonspecific protease like proteinase K could have yielded different results in our experiments, as discussed below.

In silico prediction of surface-associated proteins does not indicate which proteins are expressed and exposed on the bacterial surface. Surface-attached proteins are more challenging to work with than other protein types due to their lower abundance relative to cytoplasmic proteins, and their increased insolubility. In first-generation proteomics, which combined gel-separation with MALDI-TOF analysis, membrane proteins were underrepresented due to their low solubility. Second-generation proteomics uses gel-free techniques, analysing liquid samples with LC-MS/MS after protease treatment [204]. The surface shaving method combines two ideas: culturing live cells with proteases and identifying cleaved proteins with LC-MS/MS

after protease treatment. The technique was first used on *Streptococcus pyogenes* [213], and has since been used on various bacterial species [79, 204, 210, 214-217]. A key advantage of the surface shaving method is that it minimizes cell lysis and better represents the surface protein profile.

An advantage with our approach was that we incubated bacteria with skin cells before surface shaving. By doing this we imagined that the bacterial cells would express more biologically relevant proteins that are upregulated in response to host cells. Bacterial cells alter their gene expression in response to the environment [218]. We used a control that was treated similarly, but without the contact with human cells. By doing this we could compare the results from the two conditions. Typically, surface proteins are analysed on bacteria incubated in conventional growth medium [210, 213, 219-221], which may not induce the expression of genes responsible for infection. A limitation of incubating bacteria with human cells is the need to separate them before surface shaving to avoid contamination of human proteins. Separation on FACS is time-consuming and results in low sample throughput. Ideally, we would compare multiple clinical and commensal strains to identify differences between the groups and potential therapeutic targets.

Olaya-Abril et al. recently performed surface shaving on four *Streptococcus pneumoniae* strains under seven different culture conditions [217]. The bacteria grew in conventional medium with and without blood, in fresh or spent macrophage or A549 lung epithelial cell culture media, and in direct contact with macrophages and A549 cells for one hour. Unlike our method, they used two centrifugation steps and optical microscopy to ensure no eukaryotic cells were present. Their approach identified 279 surface proteins, where cell wall-anchored proteins were among the most frequently identified. Notably, most of the identified cell-wall proteins were not detected after contact with epithelial cells, although the significance of this observation was unclear.

We used trypsin as a protease for surface shaving, which cleaves at specific sites and generate peptide lengths ideal for mass spectrometry [204]. However, very long peptides might exceed the instrument's measurement range, and protein loops without a free end require two cleavage sites to generate a peptide [204]. To address these issues, trypsin could be replaced or combined with other proteases, such as the unspecific protease proteinase K [204, 222]. In the original surface shaving protocol by Rodriguez-Ortega et al., they used both trypsin and proteinase K.

Of 72 identified proteins, 43 were deduced from trypsin-derived peptides, 18 from proteinase K-derived peptides, and 11 from peptides derived from both enzymes. Only 4 proteins were predicted as cytoplasmic [213].

We used a lipid-based protein immobilization technology, which binds the same number of cells in each flow cell channel, and combined it with relative protein quantification. Various methods exist for this, each with their advantages and disadvantages [223]. We used TMT, a chemical label that offers higher precision than label-free quantification [223, 224].

5.1.2 Adhesion to plastic and host proteins

We examined the adhesion of *S. haemolyticus* to plastic and human extracellular matrix proteins fibrinogen, fibronectin, and collagen. These proteins provide structural support and mediate cellular processes, but also serve as binding sites for bacterial pathogens, including NAS [177-179, 225-227]. Some SRRP proteins are shown to mediate binding to host matrix proteins [87, 228, 229]. In **paper I** we compared the adhesion of ten clinical and ten commensal strains to uncoated plastic, and plastic coated with fibronectin and collagen. In **paper II** we compared the adhesion to fibrinogen and fibronectin for SraP wild type and *ΔsraP*/*ΔsecA2* mutants.

Both clinical and commensal *S. haemolyticus* strains in **paper I** adhered to plastic without significant difference between the groups. This adherence is expected due to the species' frequent biofilm formation on medical implants [31]. The adhesion to host matrix proteins in general was low for all strains in both papers. In **paper I**, the adhesion to fibronectin and collagen were significantly higher for the commensal strains compared to the clinical ones. In **paper II**, there was very low binding to fibrinogen and fibronectin and the OD levels for both wild type and mutants were around the levels of the blank. The low binding levels of *S. haemolyticus* to host matrix proteins indicate that its proteins either do not play a major role in binding or were not expressed under the experimental conditions. We used spent medium from HaCaT cells to mimic more natural growth conditions. However, this may not have triggered protein expression sufficiently. Different growth conditions, such as a skin-like medium, could have given other results [230].

One of the strains used in the fibronectin assay of **paper I** was the same as the wild type in **paper II**. However, the results varied between the experiments. The OD was above 0.2 in **paper**

I and equivalent to the blank (0.0) in **paper II**. The discrepancy could the attributed to several factors, such as different reagent batches, plate readers, and fibronectin plate suppliers. Additionally, the incubation time varied. In **paper I**, bacteria were incubated for ten hours, while in **paper II** they were incubated overnight. Variations in culturing time can influence the expression of proteins important for binding to host matrix proteins [182, 210, 231, 232].

5.1.3 Biofilm formation

Biofilm formation in *S. haemolyticus* increases its pathogenicity and antibiotic resistance due to reduced metabolic activity, making infections harder to treat [65, 98, 99]. We therefore wanted to investigate the biofilm activity of *S. haemolyticus*.

In **paper I**, we compared the biofilm formation of ten clinical and ten commensal *S. haemolyticus* strains. All strains formed biofilm, with clinical strains showing a trend towards higher biofilm formation. These strains were also part of another experiment by our group, which included 169 *S. haemolyticus* isolates. In that experiment, 67% of clinical isolates formed biofilms compared to 35% of the commensal ones, indicating that biofilm formation is an important virulence factor for this species. Additionally, biofilm-forming isolates resistant to oxacillin and aminoglycosides were most likely invasive isolates [23]. In a study of NAS isolated from central venous catheters of intensive care unit patients, *S. haemolyticus* was the predominant cause of catheter colonization, and all of them were strong biofilm formers [104]. Of the proteins identified after surface shaving, we identified several that might play a role in attachment and biofilm formation, such as Atl, serine-rich proteins, Embp, IsaA, and IsaB [74, 108, 109, 111, 193, 225, 233-237].

Previous studies have shown that SRRPs may contribute to biofilm formation [190, 238-240]. In **paper II**, we compared biofilm formation between SraP wild type and *ΔsraP*/*ΔsecA2* mutants. All strains formed biofilm, and there was no significant difference in the biofilm forming ability between wild type and *ΔsraP*/*ΔsecA2* mutants in TSB with 1% glucose.

In both **paper I** and **II**, we induced biofilm formation using TSB with 1% glucose. In **paper II**, we also used spent HaCaT medium supplemented with 10% heat-inactivated foetal bovine serum (FBS). In TSB with glucose, the wild type reached an OD_{570} value above 2 after 24 hours, while it remained below 0.5 in the spent HaCaT medium. The *Δsrap* mutant showed a slight

increase in biofilm production compared to the wild type after 24 hours in HaCaT medium, possibly due to upregulation of other surface proteins to compensate for the loss of SraP. By 48 hours, the wild type formed more biofilm than both mutants. The growth of the *S. epidermidis* positive control was markedly reduced in spent HaCaT medium with FBS. This could be a similar effect as described previously, where serum inhibits biofilm formation of this strain [241].

Various growth conditions and surfaces influence biofilm formation [67, 92, 218, 242]. For example, Qin et al. found that biofilm formation in *S. haemolyticus* was enhanced in TSB with 1% glucose, reduced in TSB with 3% NaCl, and absent in some strains in plain TSB [67]. Shrestha et al. evaluated biofilm formation in 51 clinical NAS isolates using three laboratory methods. The tissue culture method detected biofilm production in 50 isolates, the tube adherence method in 42, and the Congo red agar method in 40 [242].

Identifying the binding partner of *S. haemolyticus* SraP would clarify its role in biofilm formation. Additionally, understanding how SraP expression is regulated across different conditions and how bacteria compensate for the loss of SraP could be valuable, given that gene expression is influenced by environmental factors [218].

5.1.4 Adhesion to and survival in human cells

Bacterial adhesion to and intracellular survival in human cells are important factors for biofilm formation and development of therapy-resistant chronic infections [31, 196]. We investigated the interaction of *S. haemolyticus* with human cells. In **paper I** we analysed the adhesion of ten clinical and ten commensal strains to human keratinocytes. In **paper II**, we compared the adhesion and intracellular survival of SraP wild type and mutants (*ΔsraP/ΔsecA2*) in human keratinocytes and lung epithelial cells.

In general, there was no significant difference between the clinical and commensal strains regarding adhesion to keratinocytes in **paper I**. However, the strain used for surface shaving had the highest adhesion. Atl may facilitate low-level internalization in host cells [196], and in **paper I**, the protein was significantly more abundant after incubation with keratinocytes than in the control. However, we have not investigated the role of Atl in internalization for our strain.

In **paper II**, we observed significant differences in adhesion and survival between SraP wild type and mutants in human keratinocytes. The mutants showed over 60% reduced growth compared to the wild type in the adhesion assay and over 76% in the survival assay. The mutants also exhibited reduced adhesion and survival in lung epithelial cells. The *ΔsraP* mutant had over 50% growth reduction compared to the wild type in adhesion and survival assays, while it was over 36% for the *ΔsecA2* mutant. The results indicate that the SraP protein enhances *S. haemolyticus* adhesion and survival in these cells. Previous studies have shown that SraP in *S. aureus* facilitates binding to human platelets and lung cells, and that *sraP* expression is important for endovascular infections [86, 91, 209]. Additionally, a *S. aureus ΔsraP* mutant or antibodies towards the SraP protein reduced the adhesion to lung epithelial cells [91, 209]. Further research with the strain in a three-dimensional skin model, similar to studies on *S. aureus*, could provide additional insights into the internalization process [243].

5.1.5 Immunology

NAS can trigger the immune response or evade it through biofilm formation, capsule production, or internalization in host cells [31, 120, 121, 196]. Compared to *S. aureus* [12], NAS has fewer proteins identified for immune evasion, with biofilm formation being the primary method among NAS. Discussions on biofilm formation and internalization in host cells are covered in previous chapters.

We detected capsular proteins after surface shaving in **paper I**. Staphylococci are generally unencapsulated or have limited capsule formation [26]. In a study by Flahaut et al., they demonstrated that the polysaccharide capsule can protect *S. haemolyticus* from uptake and killing by human neutrophils, while the unencapsulated mutant showed increased biofilm formation [120]. Previous work in our group identified new capsular operons in *S. haemolyticus* [23], which have not yet been tested for their role in immune evasion. Capsular encoding genes have also been found in *S. haemolyticus* from neonatal intensive care unit patients [66], and in a global epidemiological study of the species [68]. *S. aureus* capsular polysaccharides can shield against complement binding and neutrophil phagocytosis, making them potential vaccine targets [27].

In **paper I**, the TIR domain containing protein was significantly more abundant after incubation with keratinocytes compared to the control without human cell contact. The TIR domain plays

a role in the immune systems of bacteria, plants, and animals, and is widely distributed across all domains of life. Many bacterial TIR domains are part of antiphage defence systems that kill the cell upon phage detection, protecting the community by preventing the spread of phages to neighbouring cells [244, 245]. These TIR domains are also present in the predicted phage defence systems of *S. aureus* [244]. Additionally, some *S. aureus* TIR domains have been reported to be virulence factors and can interact with the host immune response [246, 247].

In **paper II**, the *ΔsraP* and *ΔsecA2* mutants exhibited significantly lower survival in blood compared to the wild type, indicating that the SraP surface protein may shield against host immune detection and phagocytosis. In *S. aureus*, the ArlRS-MgrA signalling cascade regulates the expression of large surface-bound proteins like SraP [248-250]. These proteins can mask other surface proteins and prevent them from binding to their ligands. Inactivation of the cascade lead to a de-repression of these large proteins, leading to reduced *S. aureus* adhesion to host molecules and endothelial cells. In the *ΔsraP* and *ΔsecA2* mutants, the absence of SraP may expose other surface proteins to the immune system, resulting in increased clearance and decreased survival of the mutants in human blood.

We investigated if the *ΔsraP* and *ΔsecA2* mutants triggered an enhanced cytokine response due to the increased bacterial clearance in blood. Both mutants showed a minor, non-significant increase in the pro-inflammatory cytokine IL-8 in blood from healthy adults and immunosuppressed children. The lack of a pronounced cytokine response makes the role of SraP in immune evasion uncertain, and examining the complement response could offer more clarity. The small sample size of our experiment may have allowed individual variations to mask any differences in immune response attributable to SraP. To fully understand host-pathogen interactions, conducting animal or clinical studies is essential.

5.2 Bacteriocins

5.2.1 Romsacin, a novel bacteriocin

Several bacterial species, including NAS, can produce antimicrobial substances that inhibit the growth of competing species [65, 139]. In **paper III** we uncovered a new bacteriocin, which we have named romsacin, produced by a commensal strain of *S. haemolyticus* isolated from human skin [21, 139]. Romsacin is active against a broad range of Gram-positive bacteria. We confirmed the activity of the bacteriocin gene cluster by heterologous expression in a *S. aureus* strain that naturally lacks these genes. This resulted in the *S. aureus* strain acquiring the same antimicrobial properties as the romsacin-producing *S. haemolyticus* strain.

Romsacin is classified as a two-peptide lantibiotic, a subgroup within the lantibiotic family of bacteriocins. These lantibiotics feature two peptide components that work synergistically to enhance the antimicrobial effectiveness [155]. While two-peptide lantibiotics have been previously identified in staphylococci [251, 252], they have not been reported in *S. haemolyticus* before.

Among the 174 *S. haemolyticus* strains in our collection, we identified only one bacteriocin-producing strain. The low occurrence might be attributed to the screening methods. Failure to detect bacteriocin production does not necessarily mean it is absent. This could result from limitation of the bacteriocin webserver, the use of an insensitive species, or inadequate induction and production of the bacteriocin [167]. However, another explanation is that lantibiotic production imposes a burden for the cells [253]. Staphylococcal lantibiotics can suppress competitors, offering a competitive advantage, but their production can be costly for the producing strains, especially at high concentrations [253]. If bacteria are resistant to the bacteriocins produced by their neighbours, they can benefit from the protection these antibiotic-producing neighbours offers without bearing the costs of production themselves.

5.2.2 Antimicrobial activity

Romsacin is active against a broad range of Gram-positive bacteria including MRSA and VRE. Staphylococcal bacteriocins active against MRSA and VRE have also previously been reported [164]. MRSA and VRE are WHO priority pathogens where we urgently need new antimicrobial treatment options [166]. Another example of what romsacin inhibits are the foodborne pathogen *L. monocytogenes*, which can cause severe invasive infections [254].

As expected, romsacin showed no antimicrobial activity against the Gram-negative bacteria *E. coli*, *A. baumannii*, or *K. pneumoniae*. This is typical, as bacteriocins generally target species closely related to their producers. The outer membrane of Gram-negative bacteria acts as a barrier, preventing compounds effective against Gram-positive species from reaching their targets. However, a synergistic effect can be achieved by combining agents that disrupt the outer membrane, such as polymyxin, with compounds that are otherwise ineffective against Gram-negative bacteria [255, 256]. The lantibiotics nisin from *L. lactis* and warnerin from *Staphylococcus warneri* cannot penetrate the outer membrane of Gram-negative bacteria. However, when combined with the antibiotic polymyxin, the sensitivity of *E. coli* to these lantibiotics increases [257]. The two-peptide lantibiotic lacticin 3147, which is sequence-related to romsacin, also acts synergistically with polymyxin to inhibit Gram-negative bacteria [258]. Similarly, the spectrum of romsacin could potentially be expanded to include Gram-negative bacteria if used in combination with other compounds, although this requires further investigation.

Romsacin eradicated *S. epidermidis*, *S. haemolyticus*, MRSA, and VRE biofilms. Biofilm formation is a key virulence factor in staphylococci and enterococci, leading to infections associated with medical implants and particularly affecting patients with weakened immune systems [31, 48, 65, 259]. In mature biofilms, cells in the inner part can become dormant due to limited diffusion of oxygen and nutrients. Reduced metabolic activity and impaired penetration make cells in biofilms less susceptible to antimicrobial compounds [31, 65, 99]. Gallidermin, a lantibiotic produced by *Staphylococcus gallinarum* [260], prevents biofilm formation of *S. aureus* and *S. epidermidis* [261]. However, while romsacin eradicates established biofilms, gallidermin's activity is limited against cells within mature biofilms. The inhibitory effect of antimicrobial compounds can be enhanced through combination treatments, such as pairing the antibiotic oxacillin with the lantibiotic nisin to combat MRSA biofilm [262].

5.2.3 Mode of action

We predicted the final structure of the lantibiotic peptides based on known modifications of two other sequence-related two-peptide lantibiotics: lacticin 3147 and lichenicidin. The prediction corresponded to the MALDI-TOF MS peaks.

Lantibiotics commonly target lipid II, a precursor in peptidoglycan synthesis, which often leads to pore formation, though not in all cases. Additionally, some lantibiotics do not bind to lipid II at all, but have other mechanisms of action [263]. There are two distinct lantibiotic-lipid II binding modes: 1) the nisin-group of lantibiotics use lipid II as an anchor to form pores in the cell membrane; 2) the mersacidin-group of lantibiotics bind to lipid II differently and inhibit cell wall synthesis without inducing membrane leakage [263]. The two-peptide lantibiotic lacticin 3147, related in sequence to romsacin, follows the mersacidin binding mode. Binding of the α-peptide to lipid II does not permeabilize the target membrane, but subsequent binding of the β-peptide leads to membrane permeabilization [264]. The proposed mode of action for lichenicidin, also sequence-related to romsacin, involves the α-peptide binding to lipid II and destabilizing the membrane without forming pores. Subsequently, the β-peptide either induces pore formation independently of lipid II or is recruited by the α-peptide after its lipid II binding to initiate pore formation [265]. Gallidermin, a lantibiotic similar to nisin, interacts with lipid II but differs in the mode of action due to its shorter length (22 vs. 34 amino acids). The effectiveness of its pore formation is influenced by membrane thickness, making its interaction with lipid II the primary mechanism for bacterial killing [266, 267].

Although pore formation is a common mechanism of two-peptide lantibiotics, the propidium iodide assay on *L. lactis* revealed no pore formation by romsacin. Either romsacin does not form pores, or the method failed to detect them. One explanation could be that the pores were too small to allow propidium iodide and DNA to pass through, yet large enough for essential ions to diffuse. Propidium iodide has a molecular mass of approximately 668 g/mol [268], which is higher than that of essential ions such as sodium (23 g/mol) and potassium (39 g/mol). SEM of *L. lactis* displayed cells with normal morphology, but with striations on the cell surface, which could be a consequence of cell wall inhibition. In contrast, SEM images of *S. aureus*, *S. epidermidis*, and *S. haemolyticus* showed normal cell morphology without these striations. However, growth curves of romsacin treated *S. haemolyticus* and *S. aureus* showed a rapid antimicrobial effect within two hours. For *B. subtilis*, SEM revealed significant cell disruption, aligning with the membrane leakage observed in the membrane integrity assay. In this assay,

romsacin demonstrated an antimicrobial effect within four minutes, and measurements after one hour indicated cell death. Similar to gallidermin, romsacin's pore formation may depend on membrane thickness, which could explain the varying results across different strains. The primary mechanism for bacterial killing by romsacin may be the α-peptide's potential interaction with lipid II. Other strains and assays could have been included to determine romsacin's mode of action, for instance by measuring efflux of potassium ions or leakage of nucleic acids and proteins [269, 270]. In addition, a more certain structure of romsacin could be generated by MS/MS and determination of a three-dimensional crystal structure [271, 272].

5.2.4 Romsacin as a therapeutic agent

The activity of romsacin suggests it could be a promising antimicrobial agent. However, despite the potential of bacteriocins as antimicrobial agents, their widespread use is limited due to several factors. Below I will discuss the advantages and challenges of using bacteriocins, particularly romsacin, as therapeutic agents.

Bacteriocins offer several advantages as therapeutic agents. Their mechanisms of action often differ from traditional antibiotics [142]. Additionally, when combined with other compounds like antibiotics, bacteriocins can broaden their inhibition spectrum, prolong the lifespan of antibiotics, and reduce the development of resistance [147-151, 257, 258, 262].

Bacteriocins do not necessarily need to be purified to be beneficial, as they can also function as probiotics. Probiotics are live microorganisms, such as bacteria, that provide health benefits to the host [273]. While commonly found in fermented foods [274, 275], probiotics can also be used in other applications, including skin treatments. The skin microbiota hosts many bacteriocin-producing strains which can protect against pathogens [138]. For instance, patients with atopic dermatitis often face worsened conditions when colonized by *S. aureus* [276]. However, a study by Nakatsuji et al. showed that applying *S. epidermidis* and *S. hominis* strains with lantibiotic antimicrobial properties to the skin significantly reduced the abundance of *S. aureus* in these patients [168]. Since *S. haemolyticus* is part of the normal skin microbiota, a similar application could be feasible.

For a broader therapeutic use, bacteriocins need to be purified. Bacteriocins are typically extracted from their original producing bacteria, which often results in low yields and high

production costs. To boost production, bacteriocin genes can be heterologously expressed in faster-growing bacteria for increased yields [154, 167, 271, 277, 278], or the compounds can be chemically synthesized [279-281]. For romsacin, we know that heterologous expression in another species is possible. However, the incomplete understanding of its chemical structure complicates potential chemical synthesis. Many lantibiotics have complex structures resulting from post-translational modifications, which can pose challenges and high costs for chemical production [280].

The stability and solubility of bacteriocins are crucial for their use as therapeutic agents [142]. Romsacin is soluble in water and was purified at a 75% water concentration. It remains stable across various temperatures and pH levels. However, like most bacteriocins, it is sensitive to protease. Since bacteriocins are peptides, oral administration is problematic due to their sensitivity to proteolytic degradation in the gastrointestinal tract. This can be solved by encapsulation of the bacteriocin [142, 146]. The main types of bacteriocin encapsulation in food and pharmacy in the period 1996-2017 was film coating (50%), liposomes (23%), nanofibers (22%) and nanoparticles (4%) [282]. For instance, nisin encapsulated in solid lipid nanoparticles has demonstrated enhanced antibacterial growth inhibition and improved biofilm disruption of oral biofilms compared to free nisin [283].

Many studies on bacteriocins lack data on their safety, toxicity, and immunomodulatory effects on humans and animals [142, 146, 169]. This data is essential before proceeding to clinical trials and should also be obtained for romsacin, starting with tests on cell cultures followed by animal experiments.

Resistance mechanisms against romsacin should also be investigated. Resistance to lantibiotics can arise through various mechanisms, including modifications to the cell wall, alterations in membrane composition, expression of specific ABC transporters, and the production of resistance proteins [154, 167, 284-286].

In summary, before clinical use, romsacin's safety and efficacy need thorough investigation. The next steps include determining its structure, mechanism of action, and exploring resistance mechanisms. Additionally, both *in vitro* and *in vivo* toxicity assays and investigating immunomodulatory effects are essential before advancing to clinical trials.

5.3 Methodological considerations

Internal and external validity are two crucial aspects of research. Internal validity ensures the results are robust, confirming that they are not influenced by other factors. External validity refers to the ability to generalize the findings to other contexts beyond the specific conditions of the experiment. Many of the strengths and weaknesses of our research have been addressed in the previous sections, so the following are broader reflections.

We have ensured the internal validity by incorporating controls and using three biological and several technical replicates whenever possible. This approach ensures that our findings are robust, reproducible, and reflect a true biological variation rather than experimental error or artifacts. Additionally, to minimize the influence of external factors on sensitive assays, we have strived to use the same equipment and reagent batches and conducted the tests within a short time frame whenever feasible. For example, the varying results of the fibronectin assay for one strain between **paper I** and **paper II** could be attributed to differences in reagent batches, plate readers, plate suppliers, and incubation time. Additionally, the microbial behaviour could have changed over time due to mutations, adaptation, or environmental shifts.

An important aspect of external validity for laboratory research is the ability to generalize results to real-world settings. Microbial studies are typically conducted under controlled *in vitro* conditions or in animal models, which may not capture the complex interactions occurring within the human body. Method selection must consider simplicity, feasibility, relevance, cost, and ethics. For example, testing *S. haemolyticus* biofilm formation directly on a large human population would yield the most insightful results, but ethical constraints fortunately prevent this. Instead, we use a simpler, cost-effective laboratory method involving biofilm formation on plastic plates using specific growth media that induce biofilm formation. Although this method is less complex than animal experiments, it remains clinically relevant because *S. haemolyticus* biofilms frequently occur on plastic catheters. However, selecting an appropriate growth medium for the strains is crucial, as demonstrated in our studies.

Sample representativeness is key for external validity, and small sample sizes can limit the ability to generalize findings. For instance, results from bacterial surface shaving are specific to the tested strain, and may not represent all *S. haemolyticus* strains, although similar properties could be present in other strains. Ideally, we would compare multiple clinical and commensal strains to identify trends in the surfaceome across these groups. However, our biologically

relevant but time-consuming method limited broader comparison. Another example is the cytokine assay in **paper II** where the difficulty in recruiting immunosuppressed children led to only five biological replicates. More biological replicates could have strengthened our findings.

6 Conclusion and future aspects

S. haemolyticus naturally resides in our microbiota, but also acts as an opportunistic pathogen. Treatment can be challenging due to its biofilm production and antimicrobial resistance. The overall aim was to deepen our knowledge of virulence factors and bacteriocin production in *S. haemolyticus*.

We investigated biofilm formation and adhesion of *S. haemolyticus* to host cells and matrix proteins for clinical and commensal strains. Surface shaving was used to identify bacterial proteins expressed during interaction with human cells. We discovered both known and novel proteins that require further investigation to clarify their role in infections. Our research on the SraP protein highlighted its potential in host cell binding and immune evasion. Future studies should aim to identify SraP's binding partner and assess its effect on human immune responses.

We discovered a bacteriocin from a commensal *S. haemolyticus* strain, named romsacin after Romsa, the Sami name for Tromsø. Romsacin shows potential as an antimicrobial agent by inhibiting growth and eradicating biofilms of clinically relevant Gram-positive species, including multi-drug resistant strains of *S. haemolyticus* and *S. aureus*. Bacteriocins can enhance their inhibition spectrum and extend antibiotic lifespan when used with other compounds. However, before romsacin can be used clinically, we need to investigate its structure, mode of action, safety, efficacy, and resistance mechanisms.

Our research has advanced the understanding of *S. haemolyticus* by investigating its virulence, bacteriocin production, and human host interactions. Prior to our work, there was a knowledge gap regarding the surface proteins of *S. haemolyticus* compared to more extensively studied staphylococci like *S. aureus* and *S. epidermidis*. We identified key surface proteins after bacterial interaction with skin cells. We observed enhanced biofilm formation in clinical isolates and analysed adaptive strategies by examining blood from both immunocompromised and healthy individuals. Our research on the SraP protein revealed functional differences from other staphylococci. Additionally, our examination of bacterial interactions led to the discovery of a novel bacteriocin. These insights collectively improve our understanding of this opportunistic pathogen and could inspire more effective strategies for preventing and treating *S. haemolyticus* infections. We also hope that romsacin will become an effective antimicrobial treatment.

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Paper I

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Identification of surface proteins in a clinical *Staphylococcus haemolyticus* isolate by bacterial surface shaving

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Abstract

Background: The skin commensal *Staphylococcus haemolyticus* is an emerging nosocomial pathogen. Despite its clinical relevance, published information about *S. haemolyticus* virulence factors is scarce. In this study, the adhesive and biofilm forming properties of ten clinical and ten commensal *S. haemolyticus* strains were examined using standard adhesion and biofilm assays. One of the clinical strains was used to identify expressed surface proteins using bacterial surface shaving. Protein abundance was examined by a comparative analysis between bacterial protein expression after human keratinocyte (HaCaT) colonization and growth in cell culture media supplemented with serum. Relative protein quantification was performed by labeling peptides with tandem mass tags (TMT) prior to Mass Spectrometry analysis. Surface proteins can be used as novel targets for antimicrobial treatment and in diagnostics.

Results: Adherence to fibronectin, collagen and plastic was low in all tested strains, but with significantly higher adhesion to fibronectin ($p = 0.041$) and collagen ($p = 0.001$) in the commensal strains. There was a trend towards higher degree of biofilm formation in the clinical strains ($p = 0.059$).
By using surface shaving, 325 proteins were detected, of which 65 were classified as surface proteins. Analyses showed that the abundance of nineteen (5.8%) proteins were significantly changed following HaCaT colonization. The bacterial Toll/interleukin-1 like (TIRs) domain containing protein ($p = 0.04$), the transglycosylase SceD ($p = 0.01$), and the bifunctional autolysin Atl ($p = 0.04$) showed a 1.4, 1.6- and 1.5-fold increased abundance. The staphylococcal secretory antigen (SsaA) ($p = 0.04$) was significantly downregulated (– 1.5 fold change) following HaCaT colonization.
Among the 65 surface proteins the elastin binding protein (Ebps), LPXAG and LPXSG domain containing proteins and five LPXTG domain containing proteins were identified; three Sdr-like proteins, the extracellular matrix binding protein Embp and a SasH-like protein.

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Conclusions: This study has provided novel knowledge about expression of *S. haemolyticus* surface proteins after direct contact with eukaryotic cells and in media supplemented with serum. We have identified surface proteins and immune evasive proteins previously only functionally described in other staphylococcal species. The identification of expressed proteins after host-microbe interaction offers a tool for the discovery and design of novel targets for antimicrobial treatment.

Keywords: *Staphylococcus haemolyticus*$_1$, Surface protein$_2$, Surface shaving$_3$, biofilm$_4$, adhesion$_5$, virulence$_6$, keratinocytes$_7$, Host-microbe interaction$_8$

Background

Staphylococcus haemolyticus is a coagulase-negative staphylococcus (CoNS) and a member of the skin microbiome. It is an increasing cause of nosocomial infections associated with indwelling medical devices, particularly affecting immunocompromised patients and premature babies [1–3]. A distinct characteristic of clinical *S. haemolyticus* strains is the ability to acquire resistance to several classes of antimicrobial agents [2]. The ability to colonize and form biofilms is regarded as the most important virulence trait for CoNS [4]. Adhesion is the first step to form biofilm on surfaces [5] and staphylococci express several adhesive surface molecules that interact with eukaryotic host cell receptors, abiotic surfaces or soluble macromolecules. The number of adhesive surface proteins varies among different staphylococcal species. In *Staphylococcus aureus*, 24 different cell wall anchored proteins have been identified, while CoNS express a smaller number [6]. Cell wall anchored (CWA) proteins are covalently attached to the peptidoglycan layer. The most prevalent CWA proteins are the microbial surface component recognizing adhesive matrix molecule (MSCRAMM) family. All CWA proteins contain an LPXTG motif (Leu-Pro-X-Thr-Gly; where X can be any amino acid) that anchor the protein to the cell wall [6]. The Sdr protein subfamily of MSCRAMMs contains a serine-aspartate repeat region [1, 6] and a signal peptide with an YSIRK motif. In *S. aureus* the majority (13/21) surface proteins harbors the YSIRK/GS signal sequence, allowing delivery of surface proteins to unique locations in the cell wall [7]. *Sdr*-like genes have previously been described in *S. haemolyticus* [8].

Another family of the CWA proteins is the Serine Rich Repeats Proteins family. Like the Sdr proteins, they have a serine repeat region, but with alanine, valine or threonine instead of aspartate [9]. Bacterial surface proteins can act as new targets in treatment and prevention of infections in multiresistant bacteria. One method to examine bacterial surface proteins is by surface shaving. Surface-shaving is a technique where peptides from bacterial surface proteins are cleaved off when protease treatment is applied followed by a Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) analysis [10]. The Lipid-based Protein Immobilization (LPI™) technology enables surface shaving of intact bacterial cells in a flow cell, and thus promotes detection of proteins expressed in the surface proteome over the highly abundant cytosolic proteins. The flow cell channels, binds intact cells by a passive process. As the surface is similar in each channel, the same number of cells are bound. Thus, combining the surface shaving approach with protocols for relative quantification, such as tandem mass tags (TMT), makes studies of low abundant virulence factors possible [11–17].

Several studies on surface proteins and their relevance in host-pathogen interactions and virulence have been performed after bacterial growth in standard laboratory medium [18–22]. In order to mimic a more biological relevant host-microbe interaction, we developed a novel method to investigate expressed surface proteins of a clinical *S. haemolyticus* isolate after colonization of human keratinocytes (HaCaT) before bacterial surface shaving was performed (Fig. 1). To our knowledge surface protein shaving of bacteria subsequent to colonization of mammalian skin cells has never been described before.

In this study, we aimed to investigate the adhesive and biofilm forming abilities of ten commensal and ten clinical strains. We have previously shown that there are specific genetic signatures associated with clinical *S. haemolyticus* strains compared to commensal strains [23], thus we wanted to investigate if any functional differences in adhesive properties between commensal and clinical isolates could be observed. Furthermore, the expression of surface-associated proteins of one clinical *S. haemolyticus* strain was investigated by mass spectrometry and proteomics. The LPI surface shaving approach and relative quantification proteomics using TMT labels was employed to identify possible novel targets for treatment, prevention and biofilm formation.

Results

We wanted to examine if commensal and clinical strains had different ability to interact and adhere to selected host proteins. The adhesive ability of ten commensal and ten clinical strains to both uncoated plastic and

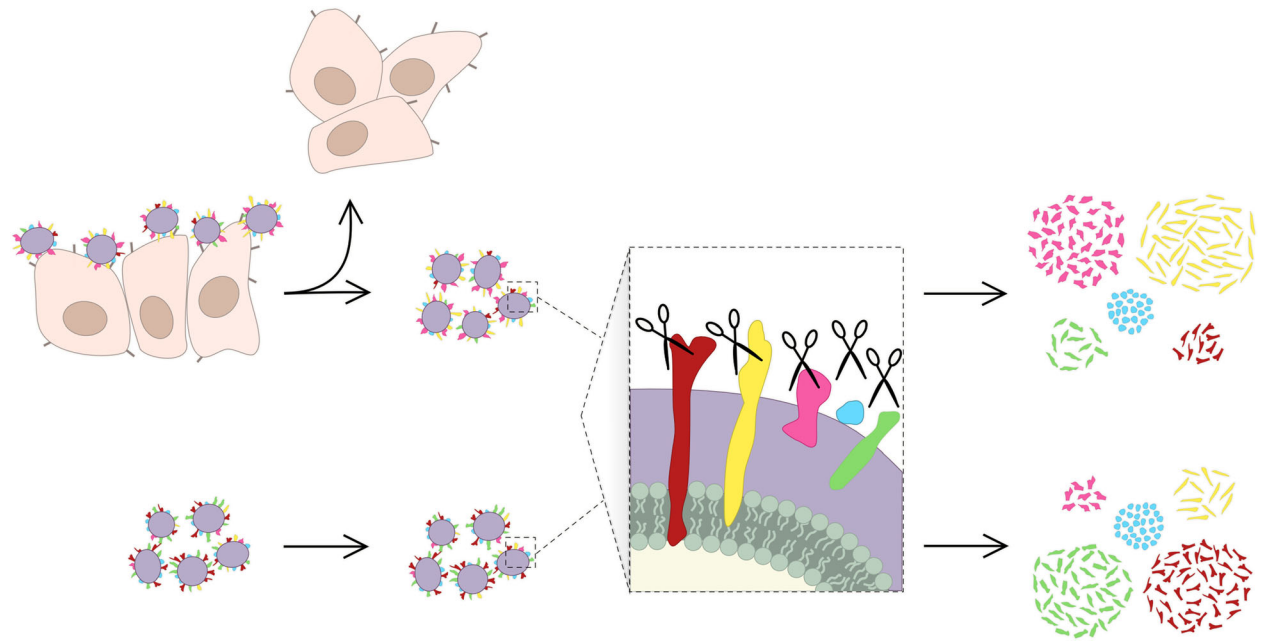

Fig. 1 Bacterial surface protein shaving, graphical abstract. Comparison of S. haemolyticus surface protein expression after HaCaT colonization (top) and the control group (bottom). Bacterial surface proteins (multicolored) are degraded by the protease Trypsin (scissors)

plastic coated with fibronectin and collagen was examined to determine if binding to fibronectin or collagen would enhance binding to plastic, as we observed that binding to plastic in its native form was generally low. Further the biofilm forming capacity was examined. Eventually, one isolate was selected for bacterial surface shaving.

Adhesion to plastic and host matrix proteins

Both clinical and commensal strains adhered to plastic but no significant difference was observed between the two groups. Fibronectin and collagen binding were low for all strains, but still significantly higher for the commensal strains compared to clinical strains, $p = 0.041$ and $p = 0.001$ respectively (Fig. 2a-c).

Semi-quantitative determination of biofilm formation

The biofilm-forming ability of the strains was determined using a semi-quantitative assay. All strains formed biofilms and a trend towards higher biofilm formation was observed for the clinical strains ($p = 0.059$) where 5/10 clinical strains formed substantial amounts of biofilm in this assay ($OD_{570} \geq 3$) compared to 0/10 commensal strains (Fig. 2d).

Adhesion to human keratinocytes

The strains were screened for their ability to adhere to human keratinocytes. In three clinical and one commensal strain > 60% of the inoculum adhered to the keratinocytes, while seven strains showed an adhesion of ~ 10–20% of the inoculum, which was in the same range as the *S. aureus* (NCTC 8325–4) control strain (Fig. 2e). On average, the clinical strains adhered better to the keratinocytes compared to the commensal strains, although the findings were not statistically significant ($p = 0.4$). One strain, displaying high adhesion to HaCaT cells in addition to being a strong biofilm producer, was chosen for further analyzes.

Bacterial surface protein shaving

Expressed surface proteins of a clinical *S. haemolyticus* isolate either colonizing HaCaT cells or grown in cell culture medium supplemented with serum, was examined by surface shaving using a Lipid-based Protein Immobilization flow cell. Relative quantification of protein abundance was performed by labelling proteins with tandem mass tags (protein markers) prior to LC-MS/MS.

Protein identification and subcellular localization of S. haemolyticus proteins detected by surface shaving

Cell surface shaving of bacteria colonizing HaCaT cells or incubated in cell culture media supplemented with serum resulted in identification of 436 proteins by LC-MS/MS analysis. Only proteins with ≥ #2 peptide-spectrum matches (PSMs) were included for further analysis, resulting in 325 proteins (Supplementary Table 1 and 2).

Subcellular localization analysis of the 325 proteins in silico and functional annotation predicted 249/325

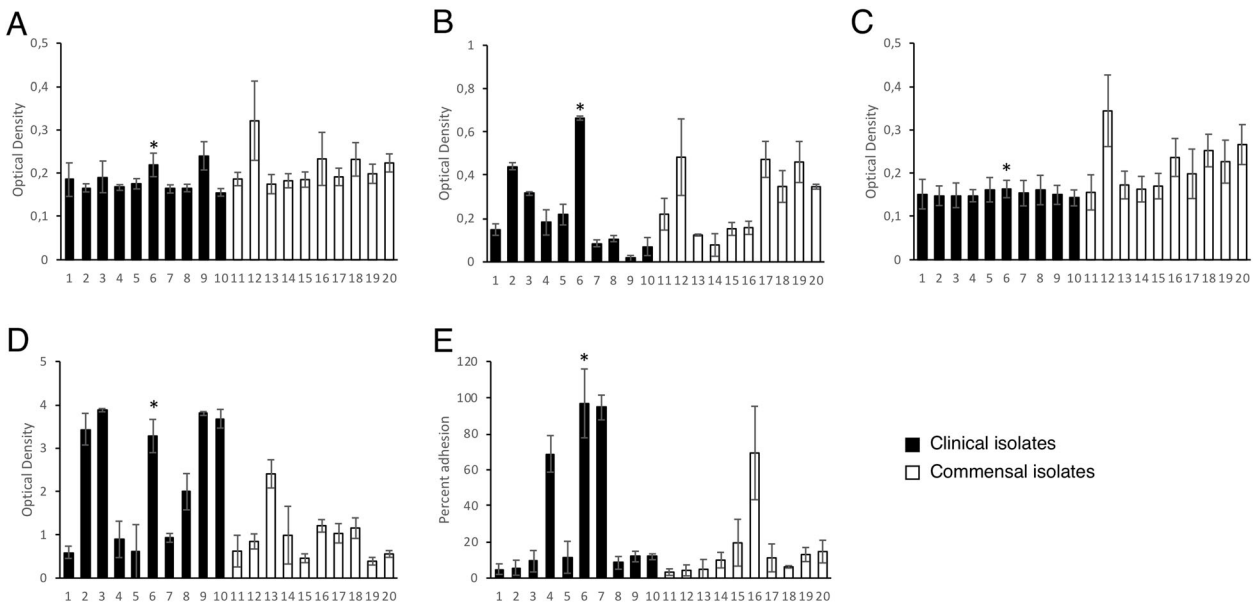

Fig. 2 Adhesion and biofilm assays of *S. haemolyticus*. Columns with black bars are clinical isolates and white bars are commensal isolates. Sample no. 6 was chosen for bacterial surface shaving (marked with asterisk). **a-c** Solid phase host matrix binding assay; **a**) Adhesion to fibronectin; **b**) Adhesion to plastic; **c**) Adhesion to collagen; **d**) Semi-quantitative determination of biofilm formation; **e**) Adhesion to human keratinocytes

(76.6%) cytoplasmic proteins, 65/325 (20.0%) surface proteins (i.e. proteins predicted to originate from the cytoplasmic membrane, cell wall or extracellular origin), and 11/325 (3.4%) as undefined proteins.

Clusters of orthologous groups

The 65 identified surface proteins were distributed in Clusters of Orthologous Groups (COG). A higher percentage of proteins in COG groups M (cell wall/membrane/envelope biogenesis) and P (inorganic ion transport and metabolism) was found when we compared the COG distribution of the identified surface proteins (65) to the COG distribution of the total number of predicted proteins (2539) encoded in the *S. haemolyticus* genome (Fig. 3).

S. haemolyticus surface proteins

Characteristic motifs of surface proteins such as signal peptides and LPXTG motifs were identified by bioinformatic tools. The covalently anchored cell wall proteins classified as MSCRAMMs are characterized by the C-terminal LPXTG sorting signal. A total of 19 proteins were predicted to have LPXTG motifs based on in silico analysis of the whole genome sequence of *S. haemolyticus* 53–38, of these seven were annotated as adhesion proteins, four were hypothetical proteins and two were DUF 402 and 368.

Of the 325 proteins identified after surface shaving, 65 were annotated as surface proteins (Table 1). Three of the LPXTG proteins identified as adhesins by the in silico analysis were expressed on the *S. haemolyticus* surface. Five LPXTG, one LPXSG and one LPXAG domain containing surface proteins were identified. Three Serine-Aspartate-Repeat (Sdr-like) proteins, the extracellular matrix binding protein (Embp), one Mannosylglucosyl-3-phosphoglycerate phosphatase (SasH-like), and two uncharacterized surface proteins were identified. Other well characterized proteins identified surface proteins were the lytic transglycosylase immunodominant staphylococcal antigen A (IsaA), the Immunodominant staphylococcal antigen B (IsaB) and the elastin binding protein (EbpS).

HaCaT colonisation causes changes in abundance of proteins

We wanted to explore if protein abundance differed when *S. haemolyticus* colonized HaCaT cells compared to when grown in cell culture media supplemented with serum. The large majority of proteins were found similarly abundant when comparing the two conditions, this included EbpS, IsaB and cytoplasmic proteins (Supplementary Table 1).

Only nineteen of 325 proteins (5.8%) showed a significant change in abundance (≥ ± 1.2 fold change) following HaCaT colonization (Table 2). The lytic transglycosylase *Staphylococcus epidermidis* D protein (SceD) ($p = 0.01$) and the autolysin Atl ($p = 0.04$) showed significantly increased abundance with a fold increase of 1.6 and 1.5

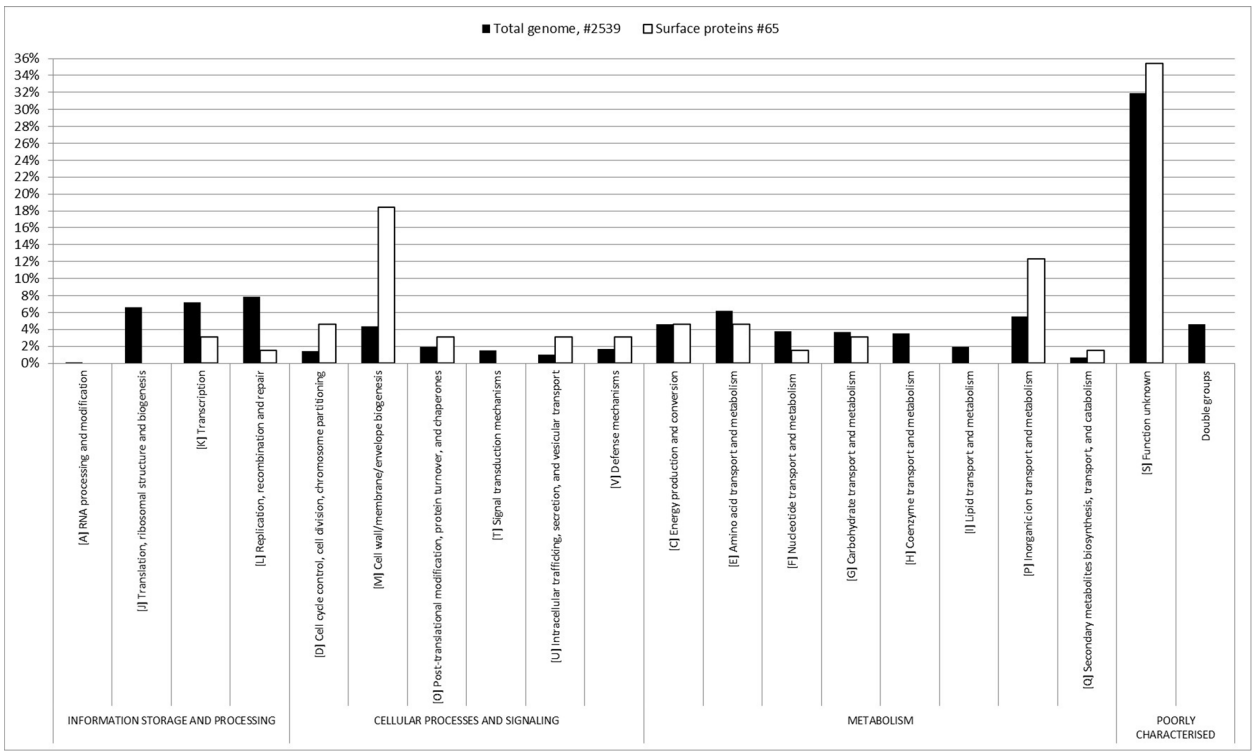

Fig. 3 Clusters of Orthologous Groups of proteins (COG). Comparison of Clusters of Orthologous Groups of proteins (COG) between the total proteins of the strain (#2539) and surface proteins (#65) found after HaCaT colonization

respectively when *S. haemolyticus* colonized keratinocytes. The Toll/interleukin-1 like (TIRs) domain protein ($p = 0.04$) also had an increase in abundance (1.4-fold) after HaCaT co-incubation, while the Staphylococcal secretory antigen (SsaA) was significantly ($p = 0.04$) less abundant following keratinocyte colonization, showing a 1.5-fold reduced abundance.

Moonlighting proteins identified by surface shaving

Several proteins that have previously been shown in other bacteria to have moonlighting functions - proteins dually engaged intracellularly and with important adhesive functions extracellularly - were found among the predicted cytoplasmic proteins. These are the moonlighting proteins glyceraldehyde-3- phosphate dehydrogenase (GAPDH), [24–26], enolase [27], aldolase (ALDA) [26], triose phosphate isomerase (TPI) [28], fructose-bisphosphate aldolase (FBA) [29], ornithine carbamoyl transferase (ARGF) [30], pyruvate kinase (PYK) [31], Inosine 5′-monophosphate dehydrogenase (IMPDH) [32], Clp [33], DNaK [34] and (Atl) [35].

Discussion

The ability to adhere to and colonize implanted biomaterials in addition to biofilm formation is considered the main virulence factors of *S. haemolyticus* and other coagulase-negative staphylococci.

[1–3]. Despite the clinical relevance of *S. haemolyticus*, published information about virulence factors is scarce compared to literature published on other staphylococcal species. We recently published a comparative analysis of clinical and commensal *S. haemolyticus* isolates [23]. We identified distinct differences in the population structure, where the clinical isolates clustered together separately from the commensal isolates. Clinical isolates were more antibiotic resistant and had different versions of genes encoding surface proteins [23]. In this study, adhesive properties and biofilm formation was compared between clinical and commensal isolates, while the expressed surface proteins were characterized in one clinical isolate after keratinocyte colonization or incubation in cell culture medium supplemented with serum.

Solid phase host matrix protein binding assay

We found that both fibronectin and collagen binding was low for all *S. haemolyticus* strains. However, fibronectin and collagen binding was significantly higher for commensal compared to the clinical strains. Fibronectin is a glycoprotein found in substantial amounts in blood and loose connective tissue [36] while collagen is an abundant class of proteins in humans, offering structural

Table 1 Predicted surface proteins after bacterial surface protein shaving of S. haemolyticus

Accession	# PSM	# Unique Peptides	Fold change HaCaT vs Control	*p*-value HaCaT vs Control	LPxTG Cell-wall anchored	Prediction of subcellular localization	Preferred name, EggNOG	Annotation summary
ACAKHAOO_00208	8	7	1.75	**0.046**	–	Extracellular (SPI)	ymaC	DUF867 type protein
ACAKHAOO_02015	7	2	1.60	**0.014**	–	Extracellular (SPI)	sceD	Putative transglycosylase SceD
ACAKHAOO_00540	433	60	1.56	0.123	LPDTG	Cell Wall (SPI)	pelX	Serine-aspartate repeat-containing protein I / YSIRK-type signal peptide-containing protein
ACAKHAOO_01033	54	25	1.46	**0.039**	–	Extracellular (SPI)	atl	Bifunctional autolysin
ACAKHAOO_00522	6	3	1.35	0.054	LPNAG	Cell Wall (SPI)	sasH	Mannosylglucosyl-3-phosphoglycerate phosphatase
ACAKHAOO_00546	188	38	1.34	0.200	LPDTG	Cell Wall (SPI)	–	Serine-aspartate repeat-containing protein I / C protein alpha-antigen
ACAKHAOO_00080	6	5	1.24	0.143	LPKSG	Cell Wall (SPI)	–	Serine-aspartate repeat-containing protein D / YSIRK-type signal peptide-containing protein
ACAKHAOO_02469	10	3	1.24	0.380	–	Extracellular (SPI)	isaA	Putative transglycosylase IsaA
ACAKHAOO_00631	5	2	1.18	0.213	–	Extracellular (SPI)	–	Hypothetical protein
ACAKHAOO_02587	2	2	1.06	0.815	–	Extracellular (SPI)	isaB	Immunodominant staphylococcal antigen B
ACAKHAOO_00744	2	2	1.01	0.916	–	Membrane (SPI)	dtpT	Di-tripepride ABC transporter
ACAKHAOO_02598	8	5	−1.01	0.997	–	Surface[a](SPII)	proX	ABC transporter substrate-binding protein / Glycine betaine/carnitine transport binding protein GbuC
ACAKHAOO_02593	81	28	−1.01	0.936	LPNTG	Cell Wall (SPI)	–	Cell wall anchor protein / hypothetical protein
ACAKHAOO_01810	3	3	−1.02	0.960	–	Membrane (SPI)	yhaN	Putative protein YhaN
ACAKHAOO_01224	2	2	−1.04	0.907	–	Membrane (SPI)	rseP	Putative zinc metalloprotease
ACAKHAOO_02549	5	3	−1.05	0.766	–	Membrane (SPI)	brpA	Polyisoprenyl-teichoic acid--peptidoglycan teichoic acid transferase TagU / transcriptional regulator
ACAKHAOO_01770	2	2	−1.06	0.763	–	Extracellular (SPI)	lytD	Bifunctional autolysin
ACAKHAOO_01453	7	3	−1.07	0.734	–	Extracellular (SPI)	lapA	Extracellular matrix-binding protein ebh / YSIRK-type signal peptide-containing protein
ACAKHAOO_01492	13	5	−1.07	0.915	–	Surface[a](Possibly sec)	ebpS	Elastin-binding protein EbpS
ACAKHAOO_01541	2	2	−1.09	0.689	–	Membrane (SPI)	yqhL	Putative protein YibN / sulfurtransferase
ACAKHAOO_00323	5	4	−1.09	0.573	–	Membrane (SPI)	ykuT	Small-conductance mechanosensitive channel
ACAKHAOO_01042	4	2	−1.10	0.582	–	Membrane (SPII)	cyoA	Putative quinol oxidase subunit 2
ACAKHAOO_	8	5	−1.10	0.528	–	Membrane (SPI)	cusA	Swarming motility protein SwrC

Table 1 Predicted surface proteins after bacterial surface protein shaving of S. haemolyticus *(Continued)*

Accession	# PSM	# Unique Peptides	Fold change HaCaT vs Control	*p*-value HaCaT vs Control	LPxTG Cell-wall anchored	Prediction of subcellular localization	Preferred name, EggNOG	Annotation summary
02168								
ACAKHAOO_01077	3	3	−1.10	0.596	–	Extracellular (SPII)	recN	Cell-wall vinding protein / hypothetical protein
ACAKHAOO_01640	2	2	−1.11	0.230	–	Membrane (SPI)	secF	Protein translocase subunit SecDF
ACAKHAOO_01808	9	5	−1.12	0.545	–	Membrane (SPII)	prsA	Foldase protein PrsA
ACAKHAOO_00719	2	1	−1.14	0.487	–	Membrane (SPII)	corC1	UPF0053 protein / HlyC/CorC family transporter
ACAKHAOO_02236	3	1	−1.14	0.633	–	Membrane (SPI)	lyrA	Lysostaphin resistance protein A
ACAKHAOO_01582	14	7	−1.16	0.360	–	Membrane (SPI)	yqfA	UPF0365 protein / hypothetical protein
ACAKHAOO_01462	6	6	−1.17	0.399	–	Membrane (SPI)	ponA	Penicillin-binding protein
ACAKHAOO_01561	3	3	−1.17	0.634	–	Extracellular (No pathway)	sodA	Superoxide dismutase [Mn/Fe]
ACAKHAOO_01806	5	3	−1.18	0.235	–	Extracellular (SPI)	yhaH	Hypothetical protein
ACAKHAOO_00464	28	12	−1.19	0.327	–	Membrane (SPI)	ftsH	ATP-dependent zinc metalloprotease FtsH
ACAKHAOO_01734	49	8	−1.19	0.394	–	Surface[a](SpI)	ytxG	DUF948 domain-containing protein
ACAKHAOO_02191	7	6	−1.20	0.452	–	Membrane (SPII)	fhuD	Ferrichrome ABC transporter substrate-binding protein
ACAKHAOO_01088	5	1	−1.20	0.591	–	Membrane (SPI)	–	DUF4064 hypothetical protein
ACAKHAOO_01722	15	8	−1.21	0.377	–	Membrane (SPI)	htrA	Serine protease Do-like HtrA/HtrB
ACAKHAOO_02068	2	2	−1.21	0.607	–	Membrane (SPI)	–	Hypothetical protein
ACAKHAOO_01403	2	1	−1.21	0.660	–	Surface[a](No pathway)	pstB	Phosphate import ATP-binding protein PstB 3
ACAKHAOO_00958	5	4	−1.22	0.357	–	Membrane (SPI)	spsB	Signal peptidase IB
ACAKHAOO_00494	2	2	−1.23	0.243	–	Membrane (SPI)	yacL	Putative PIN and TRAM-domain containing protein YacL
ACAKHAOO_02026	2	1	−1.23	0.314	–	Membrane (SPI)	atpF	ATP synthase subunit b
ACAKHAOO_01924	10	3	−1.24	0.066	–	Membrane (SPI)	–	Hypothetical protein
ACAKHAOO_01062	3	1	−1.25	0.454	–	Extracellular (No pathway)	–	Hypothetical protein
ACAKHAOO_00182	40	16	−1.28	0.265	–	Membrane (SPII)	sitA	Metal ABC transporter substrate-binding protein / Manganese-binding lipoprotein MntA

Table 1 Predicted surface proteins after bacterial surface protein shaving of S. haemolyticus *(Continued)*

Accession	# PSM	# Unique Peptides	Fold change HaCaT vs Control	*p*-value HaCaT vs Control	LPxTG Cell-wall anchored	Prediction of subcellular localization	Preferred name, EggNOG	Annotation summary
ACAKHAOO_01347	8	5	−1.29	0.151	–	Surface[a](SpI)	–	Hypothetical protein
ACAKHAOO_00718	4	3	−1.31	0.223	–	Membrane (SPI)	fruA	PTS system fructose-specific EIIABC component
ACAKHAOO_00753	25	10	−1.32	0.226	–	Surface[a](SPII)	fatB	Putative ABC transporter solute-binding protein YclQ
ACAKHAOO_01747	12	6	−1.34	0.096	–	Surface[a](SpI)	–	Hypothetical protein
ACAKHAOO_00561	8	7	−1.34	0.209	–	Membrane (No pathway)	murF	Capsule biosynthesis protein CapA
ACAKHAOO_02597	8	7	−1.35	0.204	–	Membrane (SPI)	ydfJ	Membrane protein YdfJ
ACAKHAOO_01736	2	2	−1.38	0.169	–	Surface[a](No pathway)	sftA	DNA translocase FtsK/SftA
ACAKHAOO_02099	2	2	−1.38	0.121	–	Membrane (SPII)	fecB	Iron citrate ABC transporter substrate-binding protein YfmC
ACAKHAOO_00362	6	5	−1.39	0.108	–	Membrane (SPII)	penP	Beta-lactamase
ACAKHAOO_00976	9	5	−1.40	0.051	–	Extracellular (SPII)	oppA	Oligopeptide ABC transporter / Dipeptide-binding protein DppE
ACAKHAOO_00003	25	7	−1.41	0.500	LPMTG	Cell Wall (SPI)		Hypothetical protein
ACAKHAOO_01406	17	7	−1.42	0.107	–	Membrane (SPII)	pstS	Phosphate-binding protein PstS
ACAKHAOO_02108	18	7	−1.43	0.261	–	Membrane (SPI)	–	Hypothetical protein
ACAKHAOO_00974	3	3	−1.47	0.139	–	Membrane (No pathway)	oppD	ABC transporter / nickel transport system / Oligopeptide transport ATP-binding protein OppD
ACAKHAOO_00701	11	4	−1.47	0.338	–	Surface[a](SpI)	–	Hypothetical protein
ACAKHAOO_00229	25	14	−1.49	0.130	–	Membrane (SPI)	pbpC	Beta-lactam-inducible penicillin-binding protein
ACAKHAOO_01885	2	1	−1.53	0.146	–	Membrane (SPI)	yihY	UPF0761 protein
ACAKHAOO_02197	5	2	−1.54	**0.038**	–	Extracellular (SPI)	ssaA	Staphylococcal secretory antigen SsaA / CHAP domain-containing protein
ACAKHAOO_01752	3	2	−1.67	0.102	LPNTG	Cell Wall (SPI)	–	Extracellular matrix-binding protein ebh / Signal peptide protein, YSIRK family / DUF1542
ACAKHAOO_00904	9	4	−1.74	**0.026**	–	Surface[a](SPII)	metQ	Methionine-binding lipoprotein MetQ

Surface proteins were defined as proteins predicted from cytoplasmic membrane, cell wall or extracellular origin. Positive prediction of subcellular localization was determined by a two out of three or greater concurrent results between the databases

[a]Surface: proteins were predicted as from cytoplasmic membrane, cell wall or extracellular origin, however, concurrent results between two out of three databases were not obtained

Table 2 Proteins with statistically significant altered abundance after surface shaving of S. haemolyticus incubated with human keratinocytes

Accession	# PSM	# Unique Peptides	Fold change HaCaT vs Control	p-value HaCaT vs Control	Prediction of subcellular localization	Preferred name, EggNOG	Annotation summary
ACAKHAOO_01782	3	2	1.90	0.015	Cytoplasmic	metK	S-adenosylmethionine synthase
ACAKHAOO_00208	8	7	1.75	0.046	Extracellular (SPI)	ymaC	DUF867 type protein
ACAKHAOO_02015	7	2	1.60	0.014	Extracellular (SPI)	sceD	Putative transglycosylase SceD
ACAKHAOO_02031	2	2	1.57	0.016	Cytoplasmic	upp	Uracil phosphoribosyltransferase
ACAKHAOO_00454	6	3	1.55	0.027	Cytoplasmic	ctc	50S ribosomal protein L25
ACAKHAOO_01033	54	25	1.46	0.039	Extracellular (SPI)	atl	Bifunctional autolysin
ACAKHAOO_00250	4	3	1.40	0.044	Cytoplasmic	–	TIR domain-containing protein
ACAKHAOO_00947	2	1	1.39	0.032	Cytoplasmic	ppiB	Putative peptidyl-prolyl cis-trans isomerase
ACAKHAOO_02231	2	2	1.35	0.031	Cytoplasmic	–	Putative oxidoreductase YghA
ACAKHAOO_01626	2	1	1.33	0.012	Cytoplasmic	mnmA	tRNA-specific 2-thiouridylase MnmA
ACAKHAOO_01821	4	3	1.31	0.001	Cytoplasmic	nagB	Glucosamine-6-phosphate deaminase
ACAKHAOO_00516	112	20	1.22	0.017	Cytoplasmic	tuf	Elongation factor Tu
ACAKHAOO_00797	45	14	−1.31	0.048	Cytoplasmic	pgk	Phosphoglycerate kinase
ACAKHAOO_01712	7	5	−1.44	0.026	Cytoplasmic	ezrA	Septation ring formation regulator EzrA
ACAKHAOO_01065	2	1	−1.51	0.004	Cytoplasmic	–	DUF697 domain-containing protein
ACAKHAOO_02197	5	2	−1.54	0.038	Extracellular (SPI)	ssaA	Staphylococcal secretory antigen SsaA / CHAP domain-containing protein
ACAKHAOO_01875	14	5	−1.65	0.034	Cytoplasmic	yhbO	Uncharacterized protein SH1084
ACAKHAOO_00904	9	4	−1.74	0.026	Surface (SPII)[a]	metQ	Methionine-binding lipoprotein MetQ
ACAKHAOO_01422	2	2	−1.78	0.000	Cytoplasmic	yaaN	TelA-like protein

Surface proteins were defined as proteins predicted from cytoplasmic membrane, cell wall or extracellular origin. Positive prediction of subcellular localization was determined by a two out of three or greater concurrent results between the databases

[a] Surface: proteins were predicted as from cytoplasmic membrane, cell wall or extracellular origin, however, concurrent results between two out of three databases were not obtained

support to connective tissues and the extracellular matrix [37]. In *S. aureus*, fibronectin binding is described as a crucial step in host cell adhesion. Adhesion mainly involves binding by bacterial fibronectin binding proteins (FNBPs) to fibronectin which forms a bridge between $(\alpha_5)\beta_1$ integrin on mammalian cells [38]. Low fibronectin binding in *S. haemolyticus* was previously shown when compared to *S. aureus* [39], while a varying capacity of fibronectin binding in clinical *S. haemolyticus* and other CoNS was demonstrated by Switalski et al. [40]. FnBPA and FnBPB involved in *S. aureus* fibronectin binding have not been identified in CoNS so far, but fibronectin binding by the extracellular matrix binding protein (Embp) has been shown in *S. epidermidis*. Expression of Embp in *S. epidermidis* was shown to be induced by supplementation of serum in the growth media [41]. Embp mediates adhesion to fibronectin and biofilm accumulation in *S. epidermidis* [42], and is present in

90% of clinical *S. epidermidis* strains [43]. Cell culture media supplemented with serum was also used in the adhesion assays in this study, where low binding was observed for all strains tested. We identified Embp on the surface of *S. haemolyticus* in the presence of serum. However, if Embp mediates fibronectin binding in *S. haemolyticus*, this did not result in good fibronectin binding in the adhesion assay in this study. Our findings reflect that the role of Embp in fibronectin binding of *S. haemolyticus* needs to be further investigated.

Cooperative binding of collagen in the presence of vitronectin has previously been demonstrated for *S. haemolyticus* [44]. Paulsson et al. used different bacterial growth media to induce optimal binding to both collagen and vitronectin. Thus, the type of media used in our experiments might not have been optimal for expression of proteins conferring collagen and fibronectin binding, which also could explain the low binding capacity observed in our experiments.

Adherence to plastic and semi-quantitative determination of biofilm formation

When we examined the ability to form biofilm we found trends towards more biofilm formation in the clinical strains compared to the commensal strains. However, all strains had the ability to form biofilm. In *S. epidermidis,* similar biofilm forming abilities were observed for both clinical and commensal strains, despite differences in population structure. Rather, different biofilm morphotypes and biofilm encoding genes were found among distinct genetic lineages indicating that biofilm formation is an important property of both commensal and clinical strains [45, 46].

We did not find any correlation between adherence to plastic and the degree of biofilm formation. As adherence is the first step in biofilm formation, one could expect an observed correlation between adhesion to plastic and biofilm formation. The discrepancy in these results can be explained by the use of different media when performing the two assays. It has previously been shown that the amount of biofilm varies depending on the media [47], making comparisons of results from different methods difficult.

Adhesion to human keratinocytes and bacterial surface protein shaving

We found a trend towards higher adhesion to keratinocytes for the clinical strains compared to the commensal strains. We selected one clinical strain with good adhesive and biofilm forming properties, and performed bacterial surface shaving. To date, most surface protein expression analyses are performed on bacteria incubated in bacterial growth medium [18–22]. As *S. haemolyticus* constitute a significant proportion of the skin microbiota of humans [1, 48, 49], we decided to choose a more biological relevant condition to study protein expression; incubation of *S. haemolyticus* with keratinocytes prior to bacterial surface shaving. Abundance of proteins following keratinocyte colonization was compared to protein abundance following growth in cell culture medium supplemented with bovine serum.

We identified 65 surface proteins in total, of which SceD and Atl were significantly more abundant when *S. haemolyticus* was colonizing keratinocytes. Transglycosylases cleave the β-1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine residues of peptidoglycan, accompanied with formation of 1,6-anhydromuramic acid residues [50]. In *S. aureus* the transglycosylases SceD and IsaA are well described virulence factors involved in cell wall remodeling, contributing to resistance to antimicrobial peptides, adhesion and pathogenicity, shown in a murine septic arthritis model [51]. SceD has also been shown to have a pronounced upregulation upon nasal colonization of humans and rats [51, 52].

Biofilm formation is an important virulence factor in *S. haemolyticus*, and in this study we showed a trend towards stronger biofilm formation in clinical *S. haemolyticus* isolates. The bifunctional autolysin Atl was significantly more abundant in *S. haemolyticus* colonizing HaCaT cells. Atl homologs are described in several staphylococcal species [1]. In *S. epidermidis* and *S. aureus*, Atl is important for initial adhesion and biofilm formation [53], and has in *S. epidermidis* been demonstrated to mediate adhesion to vitronectin [54]. In *S. aureus* IsaA is involved in biofilm formation and *isaA* mutants form significantly less biofilm [55]. In this study we identified IsaA when *S. haemolyticus* was grown in the presence of serum. The *S. haemolyticus* biofilm is mainly composed of environmental DNA (eDNA) and proteins [47]. As Atl also mediates adhesion indirectly by hydrolysis of the bacterial cell wall causing the release of proteins and eDNA [1], it is likely that Atl and IsaA expression also in *S. haemolyticus* have similar functions as observed in *S. epidermidis* and *S. aureus* in both adhesion and biofilm formation.

In silico analysis of the genome sequence of the clinical *S. haemolyticus* isolate used for HaCaT colonization identified 19 LPXTG containing genes. Seven of these genes were annotated as genes encoding proteins involved in adhesion, while six had unknown function. These findings resemble what is found in *S. aureus*, where 21 LPXTG genes were predicted in silico, of which eleven had unknown function [56]. In this study, five LPXTG and two LPXSG, LPXAG containing proteins were identified after surface shaving. We identified three Sdr-like proteins which were expressed both when *S. haemolyticus* were co-incubated with HaCaT cells,

and when grown in media containing serum. In *S. aureus,* transcription of SdrD and SdrG is increased in the presence of blood and serum [57, 58]. As both tested conditions contained media supplemented with serum, this could explain the expression of the Sdr-like proteins under both conditions.

In *S. epidermidis,* three Sdr proteins have been identified; SdrF, SdrG (Fbe) and SdrH. SdrF has been shown to mediate strong binding to keratins, keratinocytes and nasal epithelial cells [59]. In *S. aureus,* SdrD has been shown to mediate adhesion to keratinocytes through binding to desmoglein1, expressed in human epidermis [60]. The expression of Sdr-like proteins in *S. haemolyticus* after HaCaT colonization and grown in the presence of serum suggests that it might exert similar functions in keratinocyte binding, as found in *S. epidermidis* and *S. aureus*.

HaCaT colonization resulted in the significant upregulation of a TIR protein. TIR domain containing proteins have been shown in several pathogenic bacteria [61], but has not previously been described in *S. haemolyticus*. TirS in *S. aureus* increases survival in the host by blocking the cascade reaction leading to activation of the nuclear factor−κB (NF-κB), which regulates the expression of a pro-inflammatory immune response [62]. Bacterial circumvention of the host immune defense is an important mechanism in bacterial host colonization.

Cytoplasmic proteins

Many of the proteins identified in this experiment were predicted as cytoplasmic proteins. Detection of some cytoplasmic proteins are inevitable when performing surface shaving [10, 63]. The presence of predicted cytoplasmic proteins after bacterial surface shaving can be due to cellular lysis, moonlighting proteins or protein containing membrane-vesicles (MV) [10, 63, 64].

We recently showed that *S. haemolyticus* produces MVs [65]. The *S. haemolyticus* MV cargo mainly contained cytoplasmic proteins, amongst them several moonlighting proteins, which are proteins that express more than one function when transported to a different cellular location [24]. Release of MVs in incubation buffer after culturing and washing of cells might add to the identification of predicted cytoplasmic proteins [10].

Strengths and limitations of the study

The main advantage of the developed method is the direct contact between bacteria and mammalian cells before bacterial surface shaving, mimicking a more relevant host-microbe interaction compared to other protein expression systems. *S. haemolyticus* surface shaving subsequent to colonization of human keratinocytes has to our knowledge not been described before. By using the LPI™ approach for bacterial surface shaving, whole cells are immobilized by a passive process (personal communication Nanoxis Consulting AB) within a flow cell prior to digestion, allowing binding of intact cells only. In this study we only used one clinical isolate. In order to find surface proteins that are present only in clinical vs. commensal isolates, several isolates from different commensal and clinical lineages need to be compared.

The separation of bacteria from the mammalian cells by FACS is time consuming, leading to a low throughput of samples. The individual sorting of samples before being concentrated and subsequently subjected to surface shaving in individual LPI flow cell channels, might have led to slight variations in the concentration of cells or even slight differences in expression due to slight differences in handling time. However, we kept all samples on ice and in PBS throughout the experiment in order to minimize potential alteration of gene expression.

Conclusion

This is to our knowledge the first described study using surface shaving of expressed staphylococcal proteins after direct contact with eukaryotic cells and in cell culture media supplemented with serum. Gaining information about surface exposed proteins is important in order to better understand host-pathogen interactions, biofilm formation and for the discovery and design of novel targets for antimicrobial and anti-biofilm treatment. Thus, this method is transferable to other bacterial species and mammalian cell types. The method has provided novel knowledge about the *S. haemolyticus* surface proteins in a clinical isolate. We have identified surface proteins and immune evasive proteins previously only functionally described in other staphylococcal species. We have also identified hypothetical surface proteins, of which future analysis should be undertaken in order to describe function. Further functional assays should be performed to determine the importance of the different identified proteins in host microbe interactions and biofilm formation.

Methods

Bacterial strains and mammalian cell lines

Ten clinical and ten commensal *S. haemolyticus* strains were included in the study (Table 3). The clinical strains are a subset of a larger collection, isolated from blood, catheters and wounds [2]. The commensal strains are a subset of a collection of strains from the skin of healthy adults [49]. HaCaT cells were from a human keratinocyte cell line [66] (Cell Lines Service (CLS), Germany, no. 300493).

Table 3 S. haemolyticus strains included in the study

Sample	Country	Isolated from	Year of isolation	ENA ID[a]	Lab. ID
1	Norway	Blood	1995	ERS066267	25–12
2	Norway	Blood	2004	ERS066284	51–11
3	Norway	Blood	2002	ERS066281	51–08
4	Switzerland	Blood	2001	ERS066398	53–18
5	Germany	Blood	2008	ERS066335	53–73
6[b]	Switzerland	Wound	2004	ERS066380	53–38
7	Norway	Blood	2004	ERS066295	51–29
8	Switzerland	Blood	2004	ERS066370	53–35
9	Switzerland	Unknown	2006	ERS066381	53–49
10	Switzerland	Blood	2005	ERS066386	53–48
11	Norway	Nasal Swab	2010	ERS066315	54–64
12	Norway	Armpit	2013	ERS3370776	57–01
13	Norway	Groin	2013	ERS3370780	57–12
14	Norway	Armpit	2014	ERS3370802	57–66
15	Norway	Groin	2014	ERS3370809	58–28
16	Norway	Hamstring	2013	ERS3370784	57–22
17	Norway	Groin	2014	ERS3370790	57–33
18	Norway	Groin	2014	ERS3370800	57–61
19	Norway	Groin	2014	ERS3370806	58–08
20	Norway	Unknown	2013	ERS3370815	58–62

Ten clinical and ten commensal S. haemolyticus strains were included in the study. Samples 1–10 are clinical strains and 11–20 are commensal strains
[a]ENA = European Nucleotide Archive.
[b] Strain no. 6 was chosen for bacterial surface protein shaving

Solid phase host matrix protein binding assay

The ability of *S. haemolyticus* to adhere to collagen, fibronectin and plastic was determined using a protocol based on Edwards et al. [67]. Bacterial cultures were grown for 10 h (Optical density $(OD)_{600}$ 0.7–1.0) in Dulbecco's Modified Eagle's Medium (DMEM) (Merck, Germany) with 10% heat inactivated Fetal Bovine serum (FBS) (Thermo Fisher Scientific, MA, USA), pelleted and re-suspended to a concentration of 10^8 colony forming units (CFU)/mL. Microtiterplates (96 well) pre-coated with collagen (Thermo Fisher Scientific, MA, USA) or fibronectin, 1 μg/well (R&D Systems, MN, USA) were blocked with 150 μl 3% Bovine Serum Albumin (BSA) (Merck, Germany) for 1 h at room temperature and then washed 2x with Phosphate Buffered Saline (PBS) (Merck, Germany). Inoculum was added to plastic (CAT.NO 163320, Thermo Fisher Scientific, MA, USA), collagen and fibronectin plates and incubated for 1 h at 37 °C followed by 1x wash with PBS. The plates were fixed at 55 °C for 1 h and stained with 0.25% crystal violet (Merck, Germany) for five minutes. Biomass of adherent bacteria was determined by solubilization of crystal violet with 150 μL 70% EtOH. Absorbance (Abs) was measured at 590 nm (Versamax, Molecular Devices, CA, USA). Values from bacterial binding to wells coated with BSA only were subtracted.

Semi-quantitative determination of biofilm formation

We performed semi-quantitative determination of biofilm production as described previously [47, 68]. Biofilm formation was induced in Tryptic Soy Broth (TSB) (BD, NJ, USA / Merck, Germany) with 1% glucose (Merck, Germany) in 96-well microtiter plates (Thermo Fisher Scientific, MA, USA). All strains were tested in eight wells with three parallel runs and controls were included on each plate. After 24 h, wells were washed, fixed and stained with 0.1% crystal violet (Merck, Germany). Crystal violet was dissolved from the biofilm with 70% ethanol for 10 min and Abs_{570} was determined (Versamax, Molecular Devices, CA, USA). We removed the highest and lowest outlier for each parallel and the remaining six values were averaged. Based on the distribution of the tested strains, strains with average OD values over 1 were considered strong biofilm-producers.

Adhesion to human keratinocytes

S. haemolyticus adhesion to human keratinocytes (HaCaT) was determined. HaCaT (2×10^5 cells/ml) were

added to 24-well plates (Thermo Fisher Scientific, MA, USA) and allowed to attach for 16 h (37 °C, 5% CO_2) in DMEM with 10% FBS. Bacterial cultures were grown at 37 °C to late exponential phase (OD_{600} 0.7–1.0) in DMEM with 10% FBS, and then washed twice in Dulbecco's Phosphate Buffered Saline (DPBS) (Merck, Germany). Approximately 2×10^6 CFU in DMEM with 10% FBS were added to each well of a cell culture plate to achieve a multiplicity of infection dose (MOI) of 10:1. The plates were centrifuged at 900xG (Eppendorf 5430R, Germany) for 10 min at 37 °C and incubated for 30 min. at 37 °C in 5% CO_2 [69]. After incubation, the plates were thoroughly washed to remove all unbound bacterial cells. To enumerate the number of adhered bacteria, 0.25 mg/mL Trypsin-EDTA (Merck, Germany) and 0.1% mg/mL Triton X-100 (Merck, Germany) were added, and the suspension was pipetted in order to fully lyse the HaCaT cells. CFU/mL was determined by plating on blood agar plates (Thermo Fisher Scientific, MA, USA) and incubated at 37 °C overnight. Three biological replicates were performed.

Bacterial surface protein shaving

Preparation of bacteria for cell surface shaving

To explore the expression of surface proteins in *S. haemolyticus* when colonizing HaCaT cells, one clinical bacterial strain (53–38) with strong adhesive and biofilm-forming properties (Table 3) was co-incubated with HaCaT cells. We wanted to further explore this isolate as adhesion and biofilm formation is regarded as important virulence traits in the coagulase negative staphylococci. A bacterial control sample (same bacterial isolate) grown in cell culture media supplemented with serum but without HaCaT cultivation was included. Three biological replicates were performed for all samples and both conditions. The workflow of the bacterial surface shaving experiment is summarized in Fig. 1 and Supplementary Table 3.

HaCaT cells were seeded in 6-well plates, and bacterial cultures were grown to late exponential phase (OD_{600} 0.6 ± 0.1) in DMEM with 10% FBS, washed twice in DPBS and resuspended in DMEM with 10% FBS and further handled as previously described for the HaCaT adhesion assay. A MOI of 100:1 was used and bacteria were centrifuged with HaCaT cells for 10 min, and further incubated for 50 min. After incubation, tissue culture plates were washed 4 times with DPBS to remove free-floating bacteria. Mechanical detachment of eukaryotic and bacterial cells from the tissue culture plates was performed with a cell scraper (VWR, PA, USA) followed by pipetting in DPBS. Cells were transferred to polystyrene tubes with a cell strainer cap (Thermo Fisher Scientific, MA, USA). Twelve wells from two tissue culture plates were used for each replicate.

The samples were prepared for Fluorescence-activated cell sorting (FACS), in order to separate bacteria from HaCaT cells, by labelling with the Vancomycin BODIPY™ FL Conjugate (Thermo Fisher Scientific, MA, USA) (0.6 μg/mL), targeting the Gram-positive bacterial cell wall [70].

The bacterial control samples that were not co-cultivated with HaCaT cells were grown to late exponential phase in DMEM with 10% FBS (OD_{600} 0.6 ± 0.1) and resuspended in DPBS after centrifugation and washing and further stored on ice. Samples were then prepared for FACS by Vancomycin BODIPY™ labelling, in order to treat the bacterial control samples in a similar manner to the test samples.

Fluorescence-activated cell sorting system (FACS)

S. haemolyticus was sorted from HaCaT cells by using FACS Aria III (BD, NJ, USA) (Software BD FACSDiva 8.0.1), according to size and fluorescence. Based on the size of single bacteria (1 μm) and the fluorescent signal strength, the gating was set to sort single or doublets of bacteria. Fluorescent beads (Polystyrene Particle, Flow Cytometry grade PPS-6 K and Nano Blank Polystyrene NFPPS-52-4 K (Spherotech, IL, USA)) were used for calibration. Vancomycin BODIPY™ was excited with a 488 nm blue laser. A FITC-detector was used to read the emitted green, fluorescent light. Normal density filter 1.0 was used in front of the FSC detector. After FACS all samples were stored on ice.

Surface shaving - sample processing and generation of peptides by LPI™ HexaLane flow cell

In order to concentrate the bacterial samples after FACS (≈230 mL), samples were centrifuged twice, both steps at 10000xG for 30 min at 4 °C in swing bucket rotors (Beckman Coulter, CA, USA), resulting in samples containing approximately 2.8×10^7 CFU/mL. The concentrated samples were resuspended in ice cold PBS, kept on ice and immediately loaded into the LPI™ HexaLane Flow Cell (Nanoxis Consulting AB, Sweden), as seen in Fig. 4, step 1. To allow bacterial attachment, the flow cell was incubated for 35 min at room temperature. The cells attach to the gold coated channels in the Flow Cell by a passive process (personal communication Nanoxis Consulting AB). Unbound bacteria were removed by washing the channels with 200 μL PBS using a syringe pump (Harvard Apparatus, MA, USA) at a flow rate of 50 μL/min. Enzymatic digestion of bacterial surface proteins was performed by injecting 100 μL of trypsin (Promega, WI, USA) (40 μg/mL) into the LPI HexaLane Flow Cell channels and further incubated for 20 min at room temperature. After digestion, peptides were eluted in 200 μL PBS and the digestion was terminated by adding 4 μL formic acid (neat) (Merck, Germany). The peptide

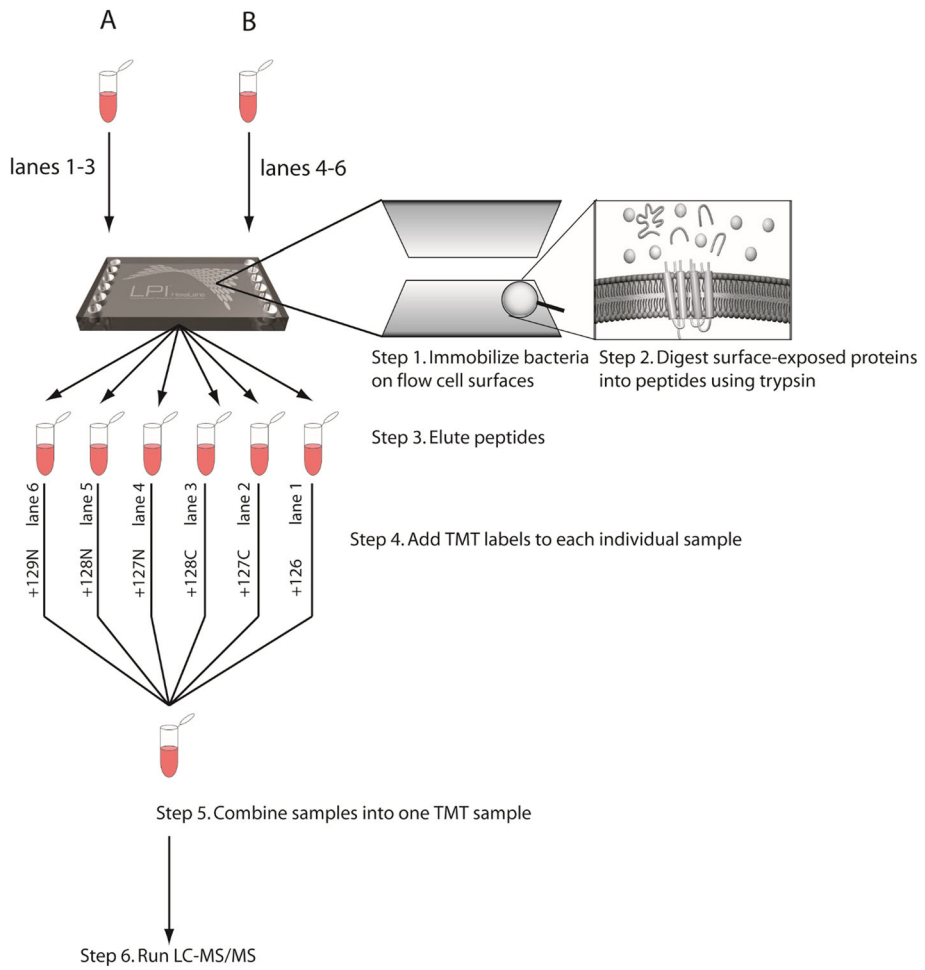

Fig. 4 The use of LPI™ methodology together with TMT labelling when performing surface shaving. Three lanes were filled with bacterial cells after exposure to HaCaT cells (**a**) and three lanes were filled with bacterial cells only exposed to media (**b**). After surface shaving, the eluted peptides were tagged with TMT labels, pooled and subsequently analyzed by LC-MS/MS.

samples were centrifuged for 10 min at 10000xG, in order to remove any cell debris and the supernatants were subsequently dried using a SpeedVac (Eppendorf, Germany) and stored at − 20 °C.

Protein identification and relative quantitation

The proteomic analysis was performed at The Proteomics Core Facility at Sahlgrenska Academy, Gothenburg University. Digested peptides were dissolved in 100 μL triethylammonium bicarbonate (TEAB) (350 mM, Thermo Fisher Scientific, MA, USA) and labelled using TMT 10-plex isobaric mass tagging reagents (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The TMT-set were fractionated into twelve fractions using Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol, but with a modified gradient (Supplementary Table 4).

The fractions were analyzed on a QExactive HF mass spectrometer (MS) interfaced with Easy-nLC1200 liquid chromatography system (LC-MS/MS) (Thermo Fisher Scientific, MA, USA). Peptides were trapped on an Acclaim Pepmap 100 C18 trap column (100 μm × 2 cm, particle size 5 μm, Thermo Fisher Scientific, MA, USA) and separated on an in-house packed analytical column (75 μm × 300 mm, particle size 3 μm, Reprosil-Pur C18, Dr. Maisch, Germany) using a gradient from 7 to 35% B over 70 min followed by an increase to 100% B for 5 min at a flow of 300 nL/min. Solvent A was 0.2% formic acid and solvent B was 80% acetonitrile, 0.2% formic acid. The instrument operated in data-dependent mode where the precursor ion mass spectra were acquired at a resolution of 60,000, the 10 most intense ions were isolated in a 0.8 Da isolation window and fragmented using collision energy HCD settings at either 28 or 50. MS2 spectra were recorded at a resolution of 60,

000, charge states 2 to 4 were selected for fragmentation and dynamic exclusion was set to 20 s with 10 ppm tolerance.

MS raw data files for the TMT set were merged for identification and relative quantification using Proteome Discoverer version 1.4 (Thermo Fisher Scientific, MA, USA). *S. haemolyticus* 53–38 with European Nucleotide Archive (ENA) accession number GCA_001226325.1 (Illumina sequence) and ENA accession number PRJEB36042 (PacBio sequence) [2] were aligned using BWA-MEM [71] and further used as reference proteome (2539 coding sequences). Structural and functional annotations were performed using Prokka [72]. Mascot 2.5 (Matrix Science Ltd., UK) was used as a search engine with precursor mass tolerance of 5 ppm and fragment mass tolerance of 200 mmu. Tryptic peptides were accepted with one missed cleavage and variable modifications of methionine oxidation, cysteine alkylation and fixed modifications of N-terminal TMT-label and lysine TMT-label were selected. Fixed Value of 13 was used for identification and the quantified proteins were filtered at 1% False Discovery Rate (FDR) resulting in a mascot score of at least 20. No missing values were present in the data set at Threshold of 2000. Proteins were grouped by sharing the same sequences to minimize redundancy. The resulting ratios were normalized in the Proteome Discoverer 1.4 and the sum of the samples cultivated with HaCaT was used as denominator. Only unique peptides were used for comparison between groups.

The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD014450.

Statistical analyses

For the results from biofilm-, solid phase host matrix protein and HaCaT adhesion assays the data were analyzed using IBM SPSS software, version 25.0. The non-parametric Mann-Whitney U-test was used to compare two groups, a p value < 0.05 was considered statistically significant.

As the technical variation for the identified proteins was assumed to be 20%, only proteins displaying a higher degree of fold change (FC) than ±1.2 were considered as biologically significant regarding increased or reduced abundance of proteins. The most changed abundance of proteins had a threshold of at least ±1.5. Welch's t-test was performed (3 parallels vs. 3 parallels) and only proteins passing filter $p < 0.05$ were considered statistically significant.

Bioinformatic analyses

LPXTG motifs were predicted in silico from the whole genome sequence of *S. haemolyticus* 53–38 using a manual sequence search. Prediction of the subcellular localization of proteins was done using PSORTb v.3.0 algorithms [73], CELLO v.2.5 [74] and LocateP v.2.0 [75]. Positive prediction of subcellular localization was determined by a two out of three or greater concurrent results between the databases. Surface proteins were defined as proteins predicted from cytoplasmic membrane, cell wall or extracellular origin.

Functional annotation of proteins was done with the EggNOG v.5.0 database with HMMER and Diamond mapping mode; i.e. functional description, seed orthologues, predicted name, KEGG KO and categorization of proteins into Clusters of Orthologous Groups of proteins (COG) [76], PHMMER v.3.3 [77, 78] and protein BLAST [79].

Moonlighting proteins were identified by using the MoonProt database and by manual searches based on published literature [80, 81].

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12866-020-01778-8.

Additional file 1: Table S1. The cell surface shaving and LC-MS/MS analysis results identified 325 proteins with ≥ #2 peptide-spectrum matches (PSMs).

Additional file 2: Table S2. FASTA sequences of the proteins from the cell surface shaving and LC-MS/MS analysis.

Additional file 3: Table S3. Workflow for bacterial protein surface shaving samples. X = performed, – = not performed

Additional file 4: Table S4. Manufacturer's and modified gradient using the Pierce High pH Reversed-Phase Peptide Fractionation Kit.

Abbreviations

Abs: Absorbance; ALDA: Aldolase; ARGF: Ornithine carbamoyl transferase; Atl: Autolysin; BSA: Bovine Serum Albumin; CFU: Colony Forming Units; CLS: Cell Lines Service; COG: Clusters of Orthologous Groups; CoNS: Coagulase Negative Staphylococci; CWA: Cell Wall Anchored; DMEM: Dulbecco's Modified Eagle's Medium; DPBS: Dulbecco's Phosphate Buffered Saline; DUF: Domain of Unknown Function; Ebps: Elastin binding protein; eDNA: Environmental DNA; Embp: Extracellular matrix binding protein; ENA: European Nucleotide Archive; FACS: Fluorescence-activated cell sorting; FC: Fold Change; FBA: Fructose-Bisphosphate Aldolase; FBS: Fetal Bovine Serum; FDR: False Discovery Rate; FNBPs: Bacterial Fibronectin binding proteins; GAPDH: Glyceraldehyde-3- phosphate dehydrogenase; HaCaT: Human Keratinocytes; IgG: Immunoglobulin G; IMPDH: Inosine 5′-monophosphate dehydrogenase; IsaA: Immunodominant staphylococcal antigen A; IsaB: Immunodominant staphylococcal antigen B; LC-MS/MS: Liquid Chromatography tandem Mass Spectrometry; LPI: Lipid-based Protein Immobilization; LPXTG: Leu-Pro-X-Thr-Gly; where X can be any amino acid; MOI: Multiplicity Of Infection; MS: Mass Spectrometry; MSCRAMM: Microbial Surface Component Recognizing Adhesive Matrix Molecule; MV: Membrane Vesicle; NF-κB: Nuclear Factor–κB; OD: Optical Density; PBS: Phosphate Buffered Saline; PRIDE: PRoteomics IDEntifications database; PSM: Peptide Spectrum match; PYK: PYruvate Kinase; SasH: Mannosylglucosyl-3-phosphoglycerate phosphatase; SceD: Lytic transglycosylase *Staphylococcus epidermidis* D protein; Sdr: Serine Aspartate repeat containing protein; SsaA: Staphylococcal secretory antigen; TEAB: Triethylammonium Bicarbonate; TIR: Toll/interleukin-1 receptor; TMT: Tandem Mass Tags; TPI: Triose Phosphate Isomerase; TSB: Tryptic Soy Broth

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Authors` contributions
RW: Participated in experimental design and planning. Performed the surface shaving experiments, analyzed the results and wrote the first draft of the manuscript. MP: Participated in experimental design and planning. Performed the adhesion experiments, analyzed the results and participated in writing the first draft of the manuscript. RK: Participated in experimental design and performance of the cell surface shaving experiment. Performed the mass spectrometry analysis and read through the final version of the manuscript. AK: Participated in experimental design and performance of the cell surface shaving experiment. Performed the mass spectrometry analysis and read through the final version of the manuscript. EF: Participated in experimental design and planning. Performed the biofilm and adhesion experiments, analyzed the results and participated in writing the first draft of the manuscript. JPC: Conceptualized the experimental design. Participated in the surface shaving and adhesion experiments, analysis of results and in writing the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials
The proteomic raw data supporting the conclusions of this article is available in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014450 [82]. The whole genome sequence of the strain used for surface shaving is depoasited in the European Nucleotide Archive with the unique identifier ERS066380 [83] and PRJEB36042 [84]. The dataset supporting the conclusions of this article is included within the article (and its Additional files S1–4).

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that the submitted work was not carried out in the presence of any personal, professional or financial relationships that could potentially be construed as a conflict of interest.
Authors AK and RK are affiliated to a company, Nanoxis Consulting AB. The Company did not have influence on the collection, analysis, or interpretation of data, the writing of the paper, or the decision to submit for publication.

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Paper II

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Staphylococcus haemolyticus SraP promotes binding to human cells

Manuscript.

Paper III

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The novel bacteriocin romsacin from *Staphylococcus haemolyticus* inhibits Gram-positive WHO priority pathogens

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The novel bacteriocin romsacin from *Staphylococcus haemolyticus* inhibits Gram-positive WHO priority pathogens

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ABSTRACT *Staphylococcus haemolyticus* is an increasingly relevant nosocomial pathogen. The combination of multi-drug resistance and ability to form biofilms makes *S. haemolyticus* infections difficult to treat. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria to inhibit growth of often closely related bacteria. Due to differences in the modes of action between bacteriocins and antibiotics, bacteriocins are normally equally potent against antibiotic-resistant and antibiotic-sensitive strains. To find bacteriocins able to inhibit *S. haemolyticus* and related species, clinical and commensal *S. haemolyticus* isolates (n = 174) were assayed for bacteriocin production. One commensal isolate produced an antimicrobial substance inhibiting *S. haemolyticus* and *Staphylococcus aureus*. The substance had physicochemical properties that are characteristic of bacteriocins. Purification, whole-genome sequencing, and mass spectrometry identified the antimicrobial as a novel two-peptide lantibiotic, hereafter named romsacin. The bacteriocin was active against a broad range of Gram-positive bacteria, such as the World Health Organization priority pathogens *S. aureus* [methicillin-resistant *S. aureus* (MRSA)] and *Enterococcus faecium* [vancomycin-resistant *E. faecium* (VRE)]. Importantly, the bacteriocin also eradicated *S. haemolyticus*, *Staphylococcus epidermidis*, MRSA, and VRE biofilms.

IMPORTANCE Bacteria produce bacteriocins to inhibit growth of other bacterial species. We have studied the antimicrobial activity of a new bacteriocin produced by the skin bacterium *S. haemolyticus*. The bacteriocin is effective against several types of Gram-positive bacteria, including highly virulent and antibiotic-resistant strains such as *Staphylococcus aureus* and *Enterococcus faecium*. Effective antimicrobials are important for the treatment of infections and the success of major surgery and chemotherapy. Bacteriocins can be part of the solution to the global concern of antimicrobial resistance.

KEYWORDS *Staphylococcus haemolyticus*, bacteriocin, antimicrobial resistance, biofilm, AMR, lanthipeptides, lantibiotics, CoNS, romsacin, WHO priority pathogens

Staphylococcus haemolyticus frequently causes hospital-acquired infections, especially affecting immunocompromised patients with indwelling medical devices (1, 2). Clinical isolates of *S. haemolyticus* are often multi-drug resistant and consequently resistant to antibiotics normally used to treat staphylococcal infections (1, 2). *S. haemolyticus* is a coagulase-negative staphylococcus (CoNS). The closely related coagulase-positive *Staphylococcus aureus* colonizes human skin and mucous membranes and is often part of the normal bacterial flora. However, the bacterium is simultaneously one of the most frequent causes of bacterial infections (1). Methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* are classified as global high priority pathogens by the World Health Organization (WHO) (1, 3–5). Vancomycin-resistant *Enterococcus faecium* (VRE) is another priority pathogen, where the acquisition of glycopeptide resistance genes and adaptation to the nosocomial setting have allowed

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it to become a successful opportunistic pathogen (4–6). It is believed that current antibacterial agents, including agents in development, are insufficient to address the rising concern of antibiotic resistance (1). A promising alternative or supplement to antibiotics is bacteriocins.

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria and typically kill closely related species. Bacteriocins can also be broad spectrum and often have a mechanism different from antibiotics (7–9). Bacteriocins are currently classified based on the presence or absence of post-translational modifications (10). Bacteriocins that are post-translationally modified belong to class I, while class II are unmodified (11–14). The lantibiotics which belong to class I are characterized by the presence of thioether cross-links between a cysteine and a dehydrated serine or threonine to form the unusual amino acids lanthionine and methyllanthionine, respectively (15). Lanthipeptide biosynthesis involves dehydration and cyclization modifications to a precursor peptide LanA, followed by proteolysis and export of the bioactive bacteriocin Lanα. Lanthipeptide gene clusters encode dedicated proteins for their biosynthesis, including LanM, which performs dehydration and cyclization, and $LanT_P$, which removes the leader sequence by proteolytic cleavage and exports it to the extracellular space (T_P: transporter and peptidase) (16). By convention, LanA liberated from its leader sequence is referred to as the pro-, core-, or mature peptide in unmodified form, although the leader is removed after the peptide is modified (5, 17). Lantibiotic producers are immune to their own bacteriocin due to the production of immunity proteins (LanI) and/or ABC transporter proteins with immunity function (LanFE/LanFEG) (4, 18). Some lantibiotics are two-peptide bacteriocins consisting of Lanα and Lanβ, derived from LanA1 and LanA2 precursor peptides, which act synergistically to exert maximal antimicrobial activity (4–6).

In this study, we investigated 174 clinical and commensal *S. haemolyticus* isolates for bacteriocin production. The aim was to find new bacteriocins able to inhibit *S. haemolyticus* and related organisms, such as *S. aureus*. One commensal isolate inhibited both species. We discovered that the genome [previously sequenced in reference (11)] of this isolate contained a lanthipeptide biosynthetic gene cluster predicted to encode a new two-peptide lantibiotic. In this work, we describe the purification and characterization of the identified two-peptide lantibiotic. The bacteriocin was active against many Gram-positive bacteria such as VRE, MRSA, and *S. haemolyticus*. In addition, the bacteriocin eradicated *S. haemolyticus*, *Staphylococcus epidermidis*, *S. aureus*, and *E. faecium* biofilms.

RESULTS

S. haemolyticus produces bacteriocins

From the collection of 174 *S. haemolyticus* isolates, overnight cultures were spotted on lawns of a clinical isolate of *S. haemolyticus* and *Staphylococcus aureus. Lactococcus lactis* was also included as an indicator due to its broad and high sensitivity toward many bacteriocins. Growth inhibition (clear zone) against indicators was observed from three of the isolates (*S. haemolyticus* 53-34, 57-27, and 58-57). Cell-free supernatants were tested, and only *S. haemolyticus* 57-27 produced an antimicrobial that was temperature stable (4°C–121°C). It was also stable to pH (2–12) but protease sensitive (trypsin), which are all characteristics of bacteriocins. *S. haemolyticus* 57-27 was isolated from the groin of an asymptomatic carrier (11, 19).

Lantibiotic genes found in *S. haemolyticus*

Assembled genomes (contigs) from 174 *S. haemolyticus* isolates were submitted to the BAGEL4 webserver to identify bacteriocin-encoding genes (20). Predicted bacteriocin gene clusters were found in all three genomes from *S. haemolyticus* isolates with antimicrobial activity. Two of the three isolates (isolate 58-57 and 53-34) were found to encode heat-labile (molecular weight >10 kDa) bacteriocins and was thus not investigated further. The remaining isolate (57-27) exhibiting inhibition contained a gene cluster

with homology to lantibiotic biosynthetic clusters. Two bacteriocin structural genes were predicted to encode the α- and β-components of a two-peptide lantibiotic with sequence homology to the A1 and A2 peptides of plantaricin W (Uniprot: D2KR94, Q9AF68). However, the two predicted core peptides shared only 67% and 51% identity to the A1 and A2 core peptides of plantaricin W, respectively. The relatively low sequence identity to known lantibiotics suggested that the cluster may encode a novel two-peptide lantibiotic (Table 1; Fig. 1). The gene product of *lanA2* is a class II lanthipeptide of the LchA2/BrtA2 family. This lanthipeptide was also uncovered during the mass screening of 100,000 RefSeq genomes done by Walker et al. (21). However, no further analysis of this bacteriocin gene cluster was done.

Annotation of the nearby genomic region revealed a complete biosynthetic gene cluster for a lantibiotic. Downstream of the bacteriocin structural genes were two genes predicted to encode lantibiotic modifying enzymes (LanM1 and LanM2) of the LanC-like super family (CDD: cl04955). Located between the two LanM genes was a gene predicted to encode a $LanT_P$ enzyme, a peptidase domain-containing ABC transporter of the SunT family (CDD: cl26602). The SunT family of peptidase exporters removes leader peptides of the double-glycine type, a common cleavage motif for bacteriocin leaders. The gene cluster found in this strain (57-27) appeared to be arranged as two operons, as no obvious immunity genes were found on the same strand as the biosynthetic genes. However, two open reading frames (ORFs) approximately 1,200 bp upstream on the opposing strand were annotated with transport/immunity function by BAGEL4. Indeed, BLAST searches resulted in matches to lantibiotic immunity ABC transporters of the MutE/EpiE family (NCBI: WP_065541939.1, E-value $2e^{-14}$). The two ORFs were, therefore, named LanFE.

We cloned genes lanA1-M2 (excluding lanE-F) into the inducible expression vector pRMC2 (22) and transformed the resulting plasmid (pRMC2_Romsacin) into *S. aureus* RN4220 by electroporation. Expression of the bacteriocin cluster was induced by adding anhydrous tetracycline (0–2 µg/mL) to the growth media of overnight cultures or of RN4220 carrying pRMC2_Romsacin. We then spotted cell-free supernatant of the overnight culture (treated at 100°C before use) onto a lawn of *Lactococcus lactis* as described in the previous section. Clear zones were observed for RN4220 expressing pRMC2_Romsacin after induction with anhydrous tetracycline concentrations of 0.08–0.12 µg/mL, but not for the wild type (no plasmid) nor for uninduced RN4220 carrying pRMC2_Romsacin.

The presence of a complete lantibiotic biosynthesis gene cluster in *S. haemolyticus* 57-27 combined with the heat stability and protease sensitivity of the antimicrobial substance strongly suggested that the strain was producing this two-peptide lantibiotic which was responsible for the antimicrobial activity. This was confirmed by heterologous

TABLE 1 Predicted bacteriocin gene cluster in *S. haemolyticus* 57-27 genome

Gene	Predicted function	Size	Homologs (GenBank)
lanF	Immunity/transport	257 aa	ATP-binding cassette domain-containing protein WP_070835451.1
lanE	Immunity/transport	232 aa	ABC transporter permease WP_070835449.1
lanA1	Core peptide	62 aa	Plantaricin C family lantibiotic WP_070835453.1
lanA2	Core peptide	67 aa	Class II lanthipeptide, LchA2/BrtA2 family WP_070835455.1
lanM1	Modification	860 aa	Type 2 lanthipeptide synthetase LanM WP_252689559.1
$lanT_P$	Transport and maturation	705 aa	Peptidase domain-containing ABC transporter WP_070835459.1
lanM2	Modification	858 aa	Lantibiotic modifying enzyme SUM61214.1

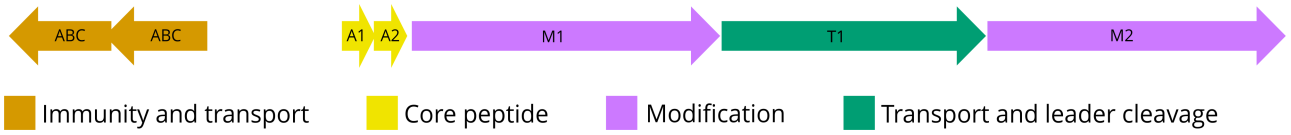

FIG 1 Bacteriocin encoding gene cluster in *S. haemolyticus* 57-27 genome. Adapted from BAGEL4.

expression of the bacteriocin cluster when induced with anhydrous tetracycline, in a different host, where it retained its ability to inhibit the *L. lactis* indicator strain.

Bacteriocin purification

We purified the bacteriocin using a standard three-step scheme consisting of ammonium sulphate precipitation followed by cationic exchange and reversed-phase chromatography (RPC). The highest antimicrobial activity against *L. lactis* was found in RPC fractions with a concentration of around 25% 2-propanol, where we could see a peak in the RPC elution profile (indicated with an arrow) (Fig. 2). We used the fractions with the highest activity for further testing, indicated by the area with the darkest gray color in Fig. 2.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

Fractions showing antimicrobial activity were pooled and analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) to confirm the identity of the purified bacteriocin. The acquired MALDI-TOF MS spectra revealed the presence of two distinct peaks at 3,149.97 m/z and 3,548.16 m/z (Fig. 3). The two smaller peaks are likely the doubly charged ions of the same molecules (3,150/2 = 1,575, 3,548/2 = 1,774). To see if the two molecules correspond to the two-peptide lantibiotic (LanA1 and LanA2) found in the genome, we performed a structure prediction for the fully modified Lanα and Lanβ peptides to calculate their expected mass.

Structure prediction

A prediction for the biosynthesis and final structures of two peptides was carried out based on the known modifications to the sequence-related lantibiotics lacticin 3147

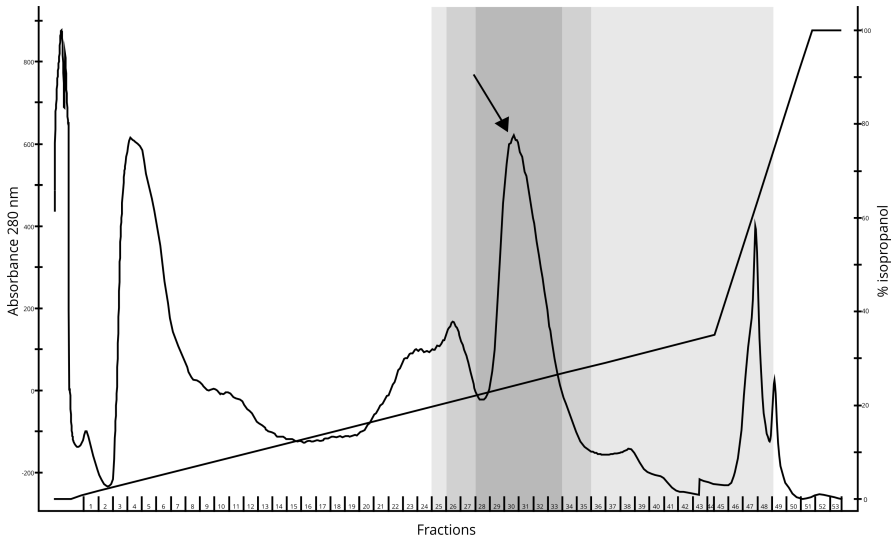

FIG 2 Reversed-phase chromatography elution profile. Antimicrobial activity was the highest in fractions eluted at approximately 25% 2-propanol (containing 0.1% trifluoroacetic acid). The area with antimicrobial activity is colored gray (fractions 25–48). The area with darkest gray color has the highest antimicrobial activity (fractions 28–33), and the peak is indicated by an arrow. The fractions with the highest antimicrobial activity were pooled for further testing.

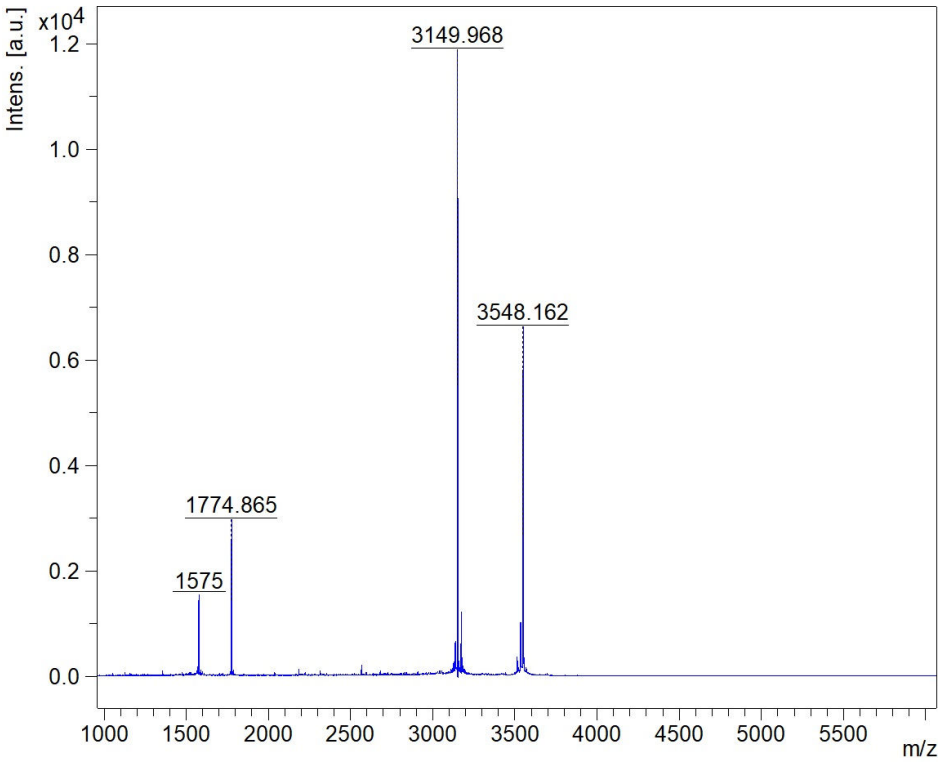

FIG 3 MALDI-TOF MS analysis of pooled active fractions obtained after RPC.

and lichenicidin. Lichenicidin A1 and A2 core peptides share 40% and 44.7% sequence identity with the LanA1 and LanA2, respectively (23) (see Fig. 4).

Using the two-peptide lantibiotics lacticin 3147 and lichenicidin as templates for structure prediction, LanA1 was predicted to have three dehydrations (−3 × 18 Da) and four reduced cysteines (−4 × 1 Da). The peptide LanA2 was predicted to have nine dehydrations (−9 × 18 Da) and four reduced cysteines (−4 × 1 Da). A typical double-glycine leader was assumed for both peptides (see Fig. 4). The resulting theoretical monoisotopic mass of the predicted Lanα and Lanβ was 3,150.3 Da and 3,548.8 Da, respectively, which corresponded well with the masses obtained by MALDI-TOF MS (3,150.3–3,149.97 = 0.33 Da, 3,548.8–3,548.16 = 0.64 Da). The predicted biosynthetic scheme is presented in Fig. 5. After having identified a new bacteriocin, we have named the bacteriocin romsacin. Consequently, the lantibiotic structural peptides LanA1 and LanA2 were designated RomA1 and RomA2 (in unmodified form) and Romα and Romβ (in modified form).

Bacteriocin antimicrobial activity

After obtaining purified romsacin, its antimicrobial spectrum against a range of Gram-negative and Gram-positive species was determined. Using a spot-on-lawn assay and planktonic growth, romsacin was shown to inhibit a broad range of Gram-positive species of both animal and human origin (Table S1; Table 2). Of potential clinical importance was the antimicrobial effect against several staphylococcal species and the WHO priority pathogens VRE and MRSA. The bacteriocin was also effective against the food-borne pathogens *Listeria monocytogenes* and *Bacillus cereus*. Gram-negative *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* strains were not inhibited by romsacin (Table S1).

S. haemolyticus, *S. epidermidis*, and *S. aureus* are often associated with biofilm-related infections from intravenous catheters, medical prostheses, and other implanted devices. For this reason, we wanted to see if romsacin was capable of disrupting biofilms formed

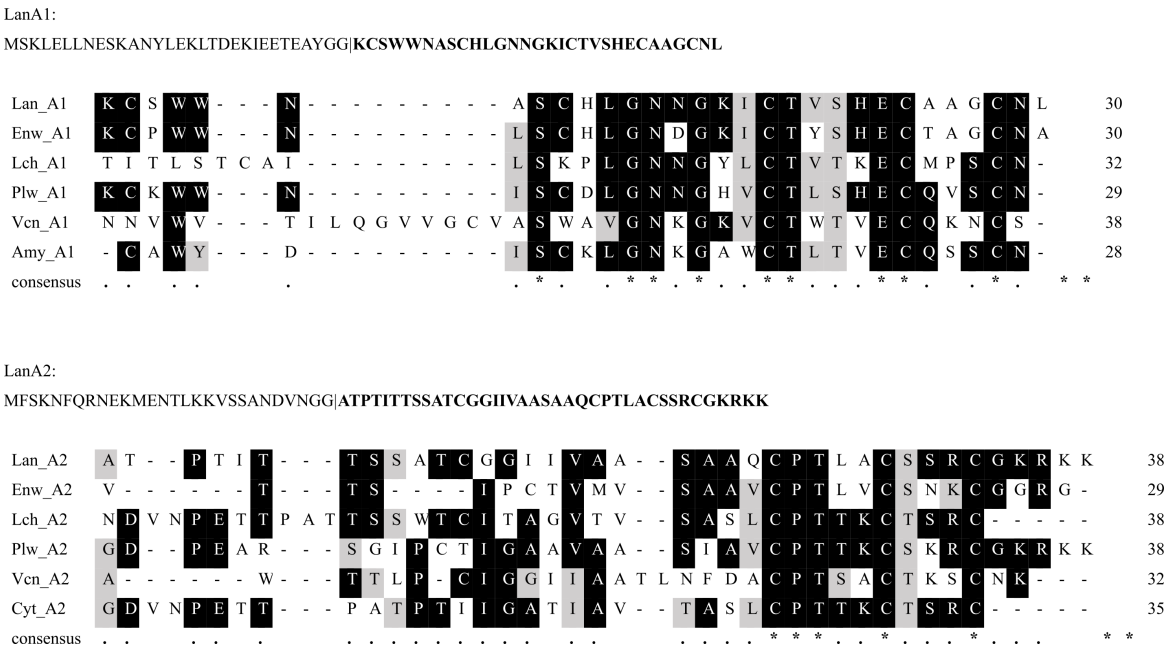

FIG 4 Multiple-sequence alignment of LanA1 and LanA2 core peptides with the core peptides of other two-peptide lantibiotics. The precursor peptide sequences are shown above with the predicted cleavage site indicated with a bar ("|") and core peptide in bold. The sequence alignment was performed using T-Coffee. Aligned sequences are the core peptides of enterocin W (Enw; H3JSS9, H3JST0) produced by *Enterococcus faecalis*, lichenicidin (Lch; P86475, P86476) produced by *Bacillus licheniformis*, plantaricin W (Plw; D2KR94, Q9AF68) produced by *Lactiplantibacillus plantarum*, vagococcin T (URZ88908.1, URZ88906.1) produced by *Vagococcus fluvialis*, amyloliquecidin (Amy; CAG7845855.1) produced by *Bacillus velezensis*, and cytolysin (Cyt; KXO02964.1) produced by *Bacillus thuringiensis*.

by these species. We also wanted to test possible biofilm disruption of *E. faecium* biofilm. By using confocal microscopy together with a live (green) and dead (red) staining technique, we could show that romsacin appeared to effectively disrupt *S. epidermidis*, *S. haemolyticus*, MRSA, and VRE biofilms. As shown in Fig. 6, the number of green cells (live) was substantially reduced following treatment with romsacin compared to untreated controls.

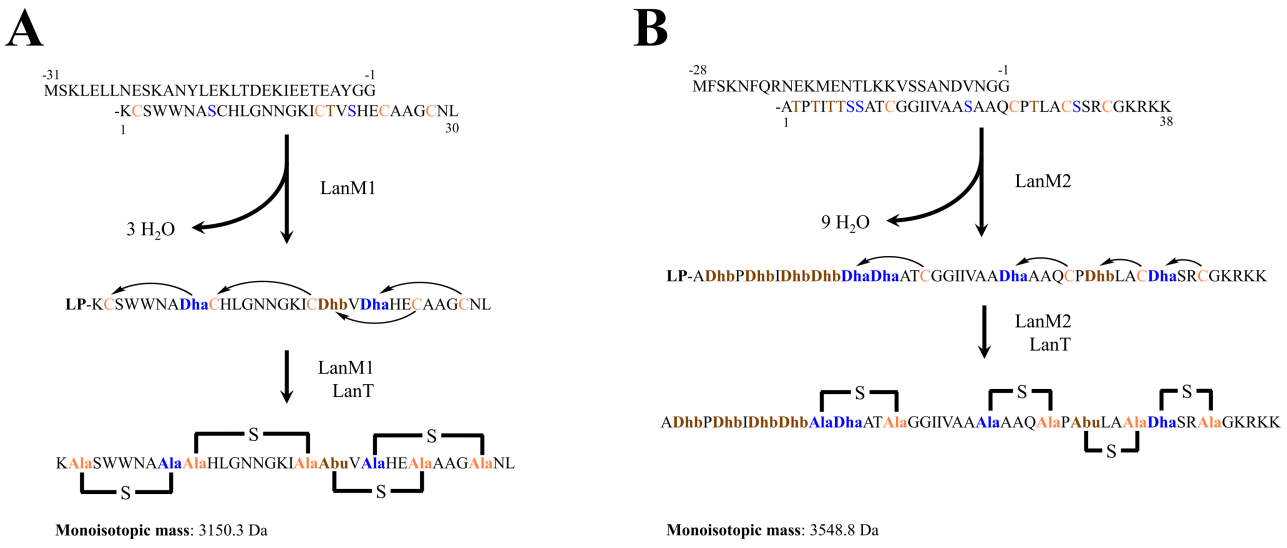

FIG 5 Predicted post-translational modifications of peptide (A) RomA1 (to produce Romα) and (B) RomA2 (to produce Romβ) and their theoretical monoisotopic mass.

TABLE 2 Romsacin inhibition against a panel of indicator strains growing on agar plates (spot-on-lawn assay) or planktonic[a]

Number	Species	Agar inhibition	Planktonic inhibition, BU/mL
1	*Lactococcus lactis* 1403 control	+++	Not tested
1	*Escherichia coli*	–	–
1	*Acinetobacter baumannii*	–	–
1	*Klebsiella pneumoniae*	–	–
1	*Enterococcus faecium* (VRE)	+++	47
2	*Enterococcus faecium* (VRE)	++	93
4	*Enterococcus faecium* (VRE)	++	47
6	*Enterococcus faecium*	+++	47
10	*Enterococcus faecium*	++	93
1	*Staphylococcus aureus* (MRSA)	++	1493
3	*Staphylococcus aureus* (MRSA)	++	93
4	*Staphylococcus aureus* (MRSA)	++	747
5	*Staphylococcus aureus* (MRSA)	++	1493
7	*Staphylococcus aureus*	+	2987
10	*Staphylococcus aureus*	++	373
1	*Staphylococcus haemolyticus*	++	93
6	*Staphylococcus haemolyticus*	++	93
7	*Staphylococcus haemolyticus*	+++	23
8	*Staphylococcus haemolyticus*	++	747
9	*Staphylococcus haemolyticus*	++	187
10	*Staphylococcus haemolyticus*	++	187
11	*Staphylococcus haemolyticus*	++	23
12	*Staphylococcus haemolyticus*	++	47
13	*Staphylococcus haemolyticus*	Not tested	47
14	*Staphylococcus haemolyticus*	Not tested	187
2	*Staphylococcus lugdunensis*	+	747
3	*Staphylococcus lugdunensis*	++	373
5	*Staphylococcus lugdunensis*	++	187
1	*Staphylococcus saprophyticus*	++	47
2	*Staphylococcus saprophyticus*	+++	12
3	*Staphylococcus saprophyticus*	++	93
3	*Staphylococcus epidermidis*	+	1493
4	*Staphylococcus epidermidis*	+	373
6	*Staphylococcus epidermidis*	Not tested	747
1	*Staphylococcus capitis*	–	1493
3	*Staphylococcus capitis*	++	93
4	*Staphylococcus capitis*	+	187
2	*Bacillus cereus*	++	187
3	*Bacillus cereus*	++	747
14	*Enterococcus faecalis*	++	747
15	*Enterococcus faecalis*	++	1493
16	*Enterococcus faecalis*	++	747
39	*Listeria monocytogenes*	++	187
40	*Listeria monocytogenes*	+	373
63	*Streptococcus uberis*	++	93

[a]Purified romsacin (3 µL) spot-on-lawn assay; no zone (–), inhibition zone 1–6 mm (+), 7–12 mm (++), and ≥13 mm (+++). Inhibition of planktonic growth is shown as the highest dilution factor that inhibited the indicator by at least 50% compared to the control with no added antimicrobial.

Pore formation assay

Propidium iodide (PI) is a fluorescent molecule where the fluorescence intensity (quantum yield) increases when intercalated in DNA. Intact bacterial cells are impermea-

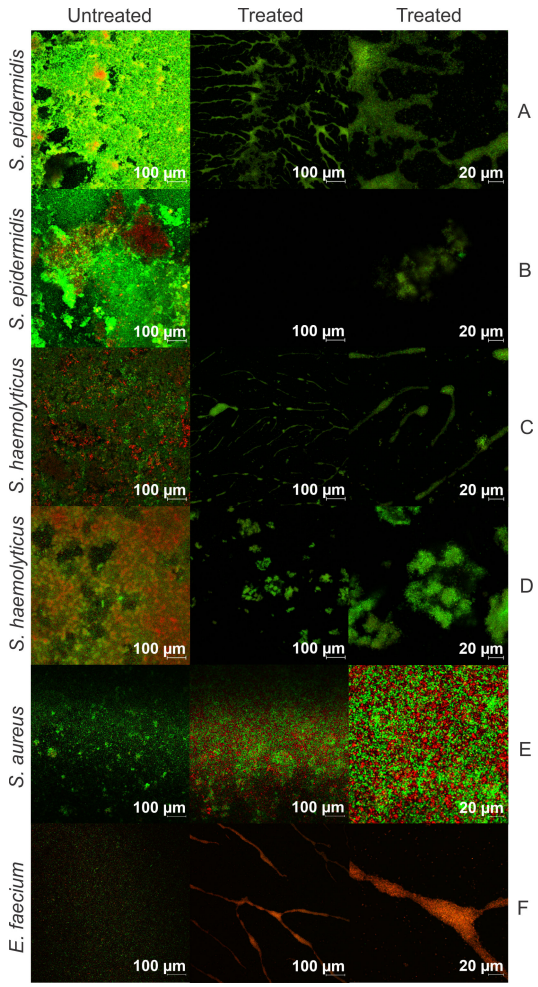

FIG 6 Biofilm confocal microscopy of (A) *S. epidermidis* no. 4, (B) *S. epidermidis* no. 6, (C) *S. haemolyticus* no. 1, (D) *S. haemolyticus* no. 6, (E) *S. aureus* no. 1 (MRSA), and (F) *E. faecium* no. 2 (VRE). Column 1 shows untreated biofilms at 100× magnification. Columns 2 and 3 show biofilms after bacteriocin treatment with 100× and 400× magnification, respectively.

ble to PI, but the molecule will diffuse into cells with a damaged membrane, resulting in an increase in fluorescence. Cells treated with romsacin in the presence of PI showed very little increase in fluorescence, with values comparable to the negative control micrococcin P1, which do not affect membrane integrity. The pore-forming bacteriocin nisin A (positive control) showed a clear increase in fluorescence as expected. The results from the assay indicated that pore formation is unlikely to be the mode of action of romsacin against *L. lactis* (Fig. 7). As we could not determine the concentration of the bacteriocins used in the assay, all bacteriocins were tested at the same antimicrobial activity expressed in bacteriocin units (BUs). A BU was defined as the amount of bacteriocin that inhibited the indicator by 50% or more in 0.2 mL of culture.

Scanning electron microscopy

The mode of action of most two-peptide lantibiotics characterized so far involves pore formation (24). As we could not see pore formation in *L. lactis* using the PI assay, we employed scanning electron microscopy (SEM) to confirm our results. Consistent with the PI assay, romsacin-treated *L. lactis* cells appeared intact (not lysed) but had a striated appearance which could not be seen in the untreated control (Fig. 8).

In order to investigate if mode of action is species dependent, we also performed SEM on MRSA, *S. haemolyticus*, *S. epidermidis*, and *Bacillus subtilis*. The integrity of

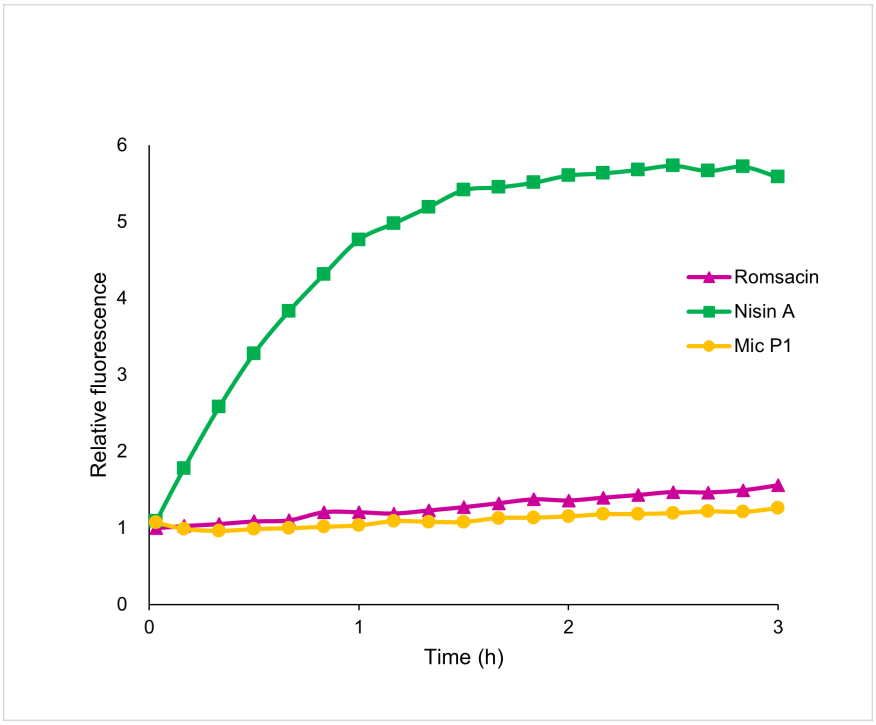

FIG 7 Propidium iodide fluorescence over time (3 hours) combined with *L. lactis* IL1403 exposed to romsacin (purple), nisin (green), and micrococcin P1 (yellow). All bacteriocins were used at 50 BUs/mL.

staphylococcal cells did not seem affected after 30 minutes with romsacin treatment. For treated *B. subtilis* samples, we saw severely disrupted cells (Fig. 8).

The SEM analyses confirmed that bacterial lysis due to pore formation is not the mode of action for the novel bacteriocin in staphylococci and lactococci.

Growth curves

The growth of both *S. haemolyticus* and MRSA treated with romsacin decreased markedly for around 2 hours (Fig. 9). After 2 hours, the growth of treated *S. haemolyticus* kept decreasing and was substantially reduced after 21 hours compared to the untreated growth control. For MRSA, the growth increased after 2 hours. The growth of non-treated MRSA increased throughout the experiment (Fig. 9).

The CFU assay was plated on agar within 1 hour after addition of bacteriocin or media to the cultures. At the start of the experiment, the CFU/mL for treated MRSA was 2.5×10^7, while for the untreated control, it was 6.3×10^7. Treated *S. haemolyticus* was 1.9×10^4 CFU/mL, while for the control, it was 5.2×10^7. The decrease in CFU, coupled with the rapid drop in optical density observed after the addition of the bacteriocin, indicates bacteriolytic effect against the majority of the *S. haemolyticus* cells. After 21 hours, CFU/mL for treated MRSA was 7.3×10^8, while for the untreated control, it was 9.3×10^8. Treated *S. haemolyticus* had 130 CFU/mL (small colony variants), while for the control, it was 1.1×10^8.

Membrane integrity assay

We investigated the romsacin effect on membrane integrity by using a *B. subtilis* strain carrying a plasmid where luciferase is constitutively expressed. If romsacin affects the permeability of the cell, D-luciferin will enter the cell, and luminescence will be emitted. ATP is needed for light to be emitted. If the cell dies, there will be a strong drop of luminescence due to lack of ATP.

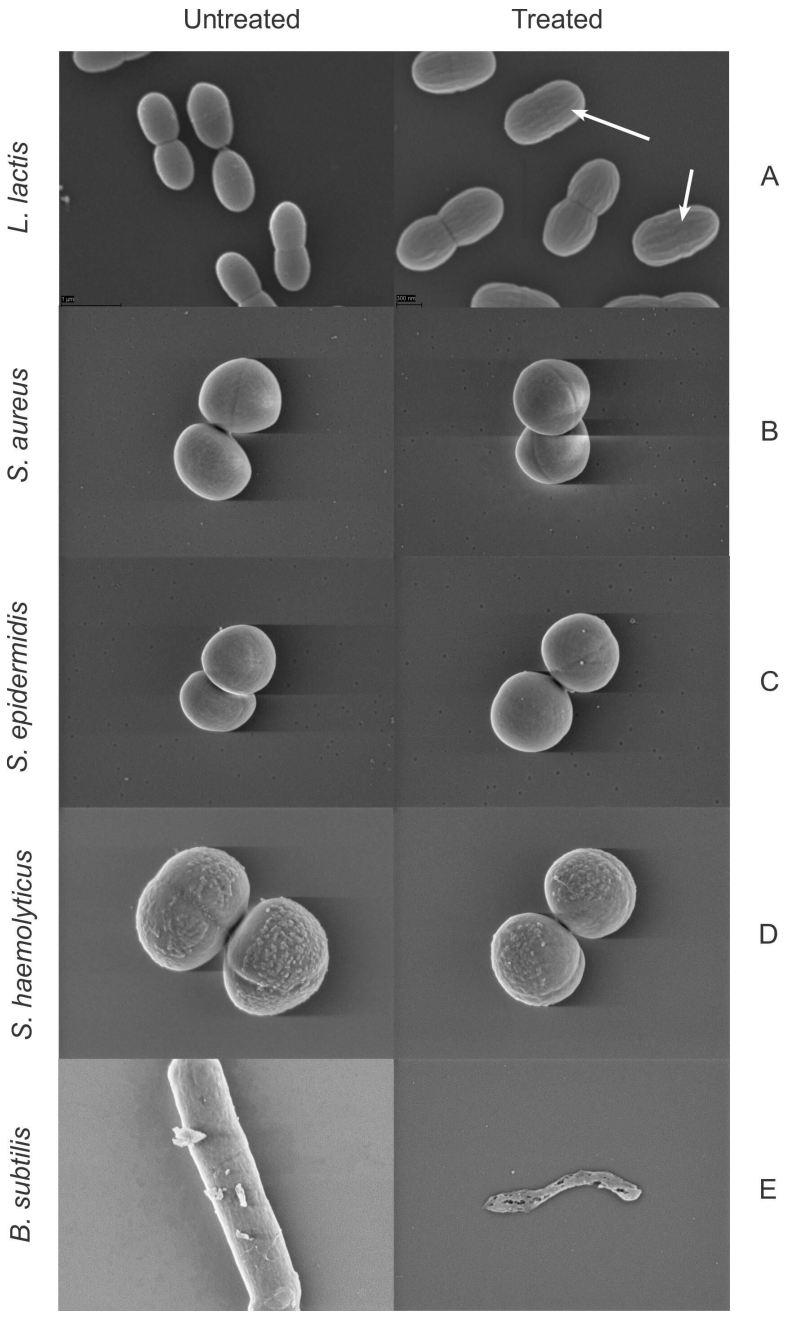

FIG 8 Scanning electron microcopy of (A) *L. lactis* IL1403, (B) *S. aureus* no. 1 (MRSA), (C) *S. epidermidis* no. 6, (D) *S. haemolyticus* no. 1, and (E) *B. subtilis* 168. All cells were exposed to bacteriocin for 30 minutes. Treated *L. lactis* cells (70,000× magnification) had a striated appearance (white arrows). The untreated *L. lactis* control is shown with a 50,000× magnification, and the staphylococci and *B. subtilis* are shown with a 40,000× magnification.

Romsacin had a quick rise in luminescence in the four first dilutions (Fig. 10), corresponding to the dilutions used in the MIC assay for *B. subtilis* (data not shown). The rise in luminescence was followed by a drop, indicating cell death. There was a clear difference in luminescence when comparing romsacin with chlorhexidine, which is known for its membrane disruptive properties (25). Chlorhexidine seems to affect the membrane faster than romsacin, as the drop in luminescence after treatment with chlorhexidine is observed immediately. For romsacin, there is a slower diffusion of D-luciferin, and it does not kill all cells during the four initial minutes. However, after

having completed all the 4-minute reads, we continued to monitor the luminescence for 10 hours to look at the long-term effect of romsacin (data not shown). At the start of the long-run experiment (within 1 hour after addition of romsacin), the relative luminescence units had dropped below 100 in the well with the most concentrated romsacin (1/20 dilution), indicating cell death.

DISCUSSION

We have identified a new bacteriocin, romsacin, produced by *S. haemolyticus*, with relatively broad antimicrobial activity. The activity was confirmed by heterologous expression of the bacteriocin gene cluster in a different host. Two-peptide lantibiotics have previously been described in staphylococci (26, 27), but we believe this is the first description of a two-peptide lantibiotic in *S. haemolyticus*. The bacteriocin romsacin is active against a broad range of Gram-positive bacteria, including the WHO priority pathogens MRSA and VRE. The pathogens on the WHO priority list have been reported as a global health threat where we urgently need new antimicrobial treatment options (6). Several reports describe bacteriocins effective against MRSA and VRE (7, 28–30). Romsacin belongs to the lanthipeptides. Some, but not all, bacteriocins within that group are effective against MRSA (7). As different clinical strains have different resistance profiles, it is important to map out several possible therapeutic alternatives.

CoNS is part of the microbiota of skin and mucous membranes of humans and animals, and production of bacteriocins by CoNS is well known. However, the biological role of bacteriocins in host colonizers is not known, but findings suggest that bacteriocins promote host colonization by eliminating competitors (31–33). Several staphylococcal species produce bacteriocins, named staphylococcins, where the majority are classified as lantibiotics (34, 35). Six well-characterized bacteriocins have been described for *S. epidermidis*, and several staphylococcins have been shown to exert inhibitory activity against *S. aureus* and have a potential as treatment option to staphylococcal or other Gram-positive bacterial infections (34). Bacteriocin production by staphylococcal species inhabiting the human nose showed activity against several bacterial species in the nasal microbiota, such as *Moraxella catarrhalis* (36). A few publications describe bacteriocin production in *S. haemolyticus* from animal origin (7–9). One of the studies describes a *S. haemolyticus* bacteriocin with activity against a mastitis-related *S. aureus* strain (9). Romsacin is the first description of a bacteriocin from a commensal *S. haemolyticus* isolated from humans.

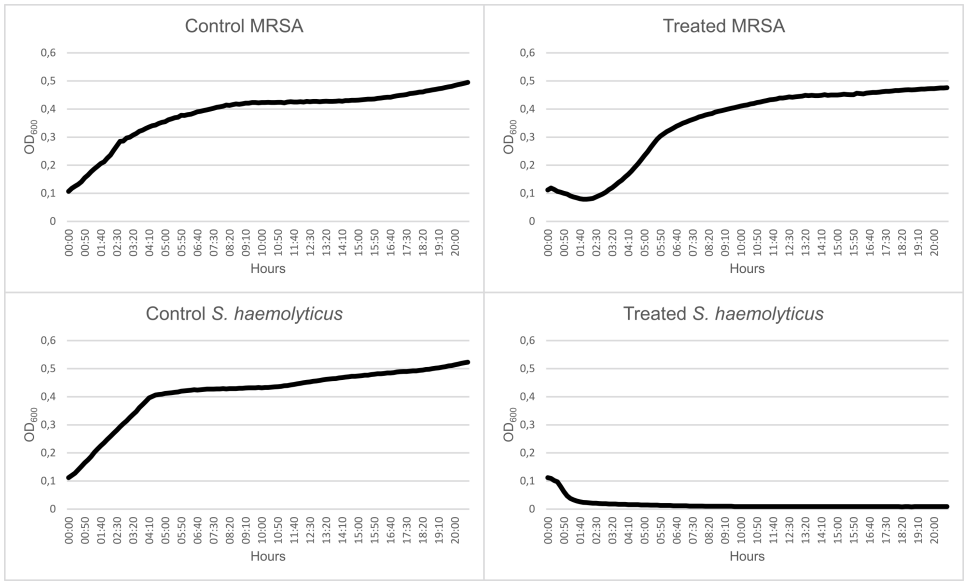

FIG 9 Growth curve 0–21 hours of *S. aureus* MRSA (no. 1) and *S. haemolyticus* (no. 1) untreated or treated with romsacin.

Romsacin had no effect against *E. coli*, *A. baumannii*, or *K. pneumoniae*, as bacteriocins originating from Gram-positive bacteria are usually not effective against Gram-negative bacteria. However, some studies report that bacteriocins from Gram-positive bacteria can gain activity and act synergistically with other compounds known to inhibit growth or permeabilize the outer membrane of Gram-negative bacteria (37, 38). Nisin has been shown to be active against *E. coli* (39) and *Pseudomonas aeruginosa* when combined with outer membrane permeabilizer polymyxin B nonapeptide (PMBN) or metal ion chelator EDTA (40, 41). Similarly, the spectrum of activity of romsacin could potentially be expanded to include Gram-negative bacteria if used in combination with other compounds such as PMBN and EDTA. However, this remains to be investigated.

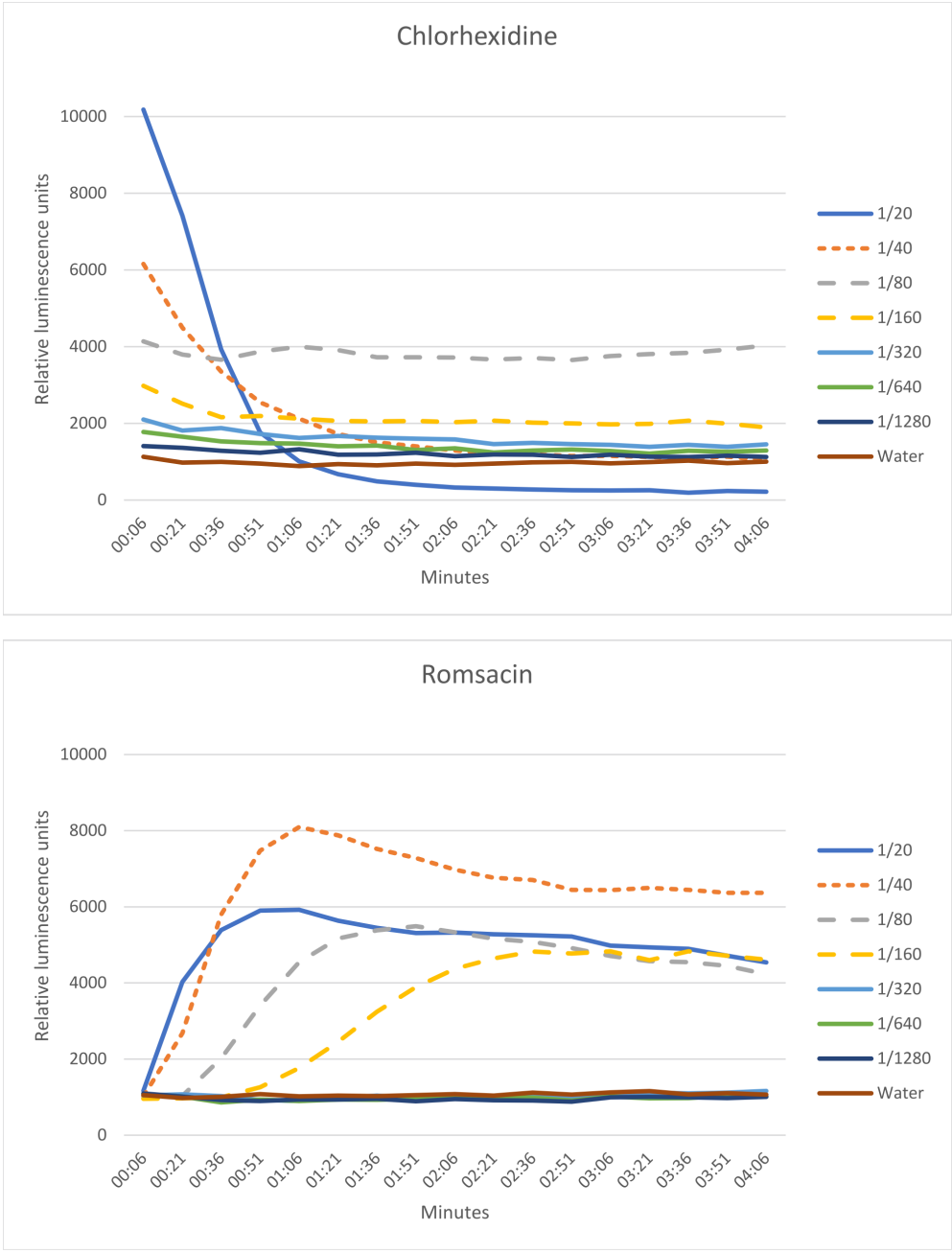

FIG 10 Membrane integrity assay with *B. subtilis* 168 carrying the pCSS962 plasmid. The bacteria were treated with either chlorhexidine or romsacin, and luminescence was measured for 4 minutes. Seven dilutions of the antimicrobial compound were used (1/20 to 1/1,280) in addition to water. Readings were made 0–4 minutes after addition of chlorhexidine or romsacin.

Romsacin effectively eradicated the *S. epidermidis*, *S. haemolyticus*, MRSA, and VRE biofilms. Biofilm formation is a major virulence factor among staphylococci and enterococci, causing infections associated with foreign body surfaces, especially affecting patients with weakened immune systems (31, 32, 42–44). Microbial cells in biofilms are less susceptible to antibiotics than planktonic cells, caused by reduced metabolism and impaired diffusion/penetration of antibiotics (31, 43, 45, 46). Romsacin was shown to effectively disrupt both *S. haemolyticus*, *S. epidermidis*, MRSA, and VRE biofilms. However, fluorescent signals in treated samples of *S. epidermidis*, *S. haemolyticus*, and *E. faecium* were low, indicating a loss of biofilm/bacteria following treatment. The loss of biofilm was not of the same extent in the romsacin-treated *S. aureus* sample, but the number of live cells was markedly reduced compared to the control. Bacteria that have formed biofilms often have 10 to 1,000 times higher tolerance to antibiotics compared to planktonic cells (39). The bacteriocin gallidermin produced by *Staphylococcus gallinarum*, efficiently eradicated biofilms formed by *S. epidermidis* and *S. aureus* (47). Different bacteriocins have been shown to have various antibiofilm strategies, making them attractive candidates for biofilm eradication (48). As there are few effective treatment options against biofilms, new additions, such as romsacin, are needed.

Bacteriocins produced by staphylococci are commonly encoded on plasmids or other mobile genetic elements such as transposons but can also be chromosomally encoded (34). Lantibiotic gene clusters acquired by horizontal gene transfer have previously been described in *S. haemolyticus* strains originating from rice seeds (49). The prevalence of bacteriocin gene clusters on mobile genetic elements could suggest that they provide a benefit to their host. The romsacin gene cluster is located on a contig which has features indicating that it is part of a plasmid. Downstream of the romsacin gene cluster is a *repA* gene which initiates replication of plasmids. Also located in the same genomic region is a Tn552 DNA invertase gene and an IS6 family transposase, suggesting that the bacteriocin is likely part of a mobile genetic element.

The structure of romsacin was not determined experimentally with much certainty (by, e.g., MS/MS or crystal structure). However, lantibiotics that bind to lipid II contain a conserved lipid II binding motif GxxxTx(S/T)x(E/D)C (50). The (methyl)lanthionine ring structures form a defined binding pocket for lipid II and are, therefore, relatively predictable (51); the same motif is present in RomA1. This leaves few options for the remaining cysteines and serines/threonines (Ser/Thr). Although a varying number of Ser/Thr can remain unmodified in the final structure, the mass difference of 18 Da (corresponding to water) will correspond to the number of modified Ser/Thr. The β-peptide of two-peptide lantibiotics show much less homology to each other than the α-peptides, but many have a CPTxxCxxxC motif at the C-terminal end (52). Mutations introduced to alter the ring structures of the β-peptide of lacticin 3147 were inactive or not processed by the cognate LanM (53). This suggests that the ring structures of the β-peptides are also well conserved, despite much less being known about their role/function. By applying modifications consistent with lantibiotics to the two predicted lantibiotic precursors found in the genome, we obtained expected masses that almost exactly matched those obtained by MALDI-TOF MS. Taken together, we are confident the purified bacteriocin is derived from *romA1* and *romA2*.

Most lantibiotics have been shown to bind the cell wall synthesis precursor molecule lipid II. Among the single-peptide lantibiotics, two different but overlapping modes of action have been described (24). The type-A(I) lantibiotics such as nisin first interact with lipid II, thereby disrupting cell wall synthesis, but will subsequently insert into the membrane and aggregate into a pore complex (24). Nisin exposure causes leakage of intracellular contents (54). Lantibiotics of type-A(II) and type-B have not been shown to form pores but kill target cells by inhibition of cell wall synthesis and likely additional unknown factors (24). Two-peptide lantibiotics are believed to use the dual mode of action only, where the α-peptide forms a complex with lipid II which recruits the β-peptide to form a pore (23). The propidium iodide pore formation assay has been used previously to examine the mode of action of bacteriocins, including two-peptide

lantibiotics (55, 56). The mode of action of the bacteriocin vagococcin T, with sequence homology to romsacin (Fig. 4), is by forming pores in the bacterial cell membrane (55). However, we were not able to measure any pore formation in *L. lactis* using this assay. It could be that romsacin forms pores too small for the passage of PI and/or DNA but still permits the diffusion of essential ions such as H^+, K^+, and PO_4^{3-}, which leads to loss of turgor pressure. SEM micrographs of *L. lactis* showed cells of normal morphology, except all cells showed striations (lines) on the surface perpendicular with the septum that were not present in the control. The underlying peptidoglycan architecture of *L. lactis* is parallel to the septal plane, opposite of the striations (57). The striated appearance is likely a consequence of cell wall inhibition; however, we have not been able to explain its cause or structure. SEM micrographs of *S. aureus*, *S. epidermidis*, and *S. haemolyticus* also showed cells with normal morphology. Increased incubation time could have given other results and should be tested in the future. For *B. subtilis,* massive cell disruption was observed, which correlates well with the membrane integrity assay, where the romsacin-treated *B. subtilis* reporter strain showed rapid membrane leakage. Growth curves of romsacin-treated *S. haemolyticus* and *S. aureus* cells showed a rapid antimicrobial effect within 2 hours. This indicates that the bacteriocin has a bacteriolytic effect (58, 59). After 2 hours, the *S. aureus* cells regain growth, which displays single-cell resistance against romsacin, which can be explained by a heterogenous population (58). The confocal images of the *S. aureus* biofilms also showed that not all cells in the biofilm were eradicated to the same extent as it was observed for *S. haemolyticus* and *E. faecium*, supporting the single-cell resistance observed also in the growth curve. Combination treatment using romsacin and a second antimicrobial agent should, therefore, be tested in the future.

Conclusion

In this study, we describe a new bacteriocin, romsacin, found in a commensal *S. haemolyticus* isolate. The bacteriocin has broad antimicrobial activity, both against planktonic cells and bacterial biofilms. Romsacin is a promising contributor to combat antibiotic-resistant pathogens. Further work is needed to establish the therapeutic potential of romsacin, both alone and in combinations with other compounds, and to determine its structure and mechanism of action.

MATERIALS AND METHODS

Detecting bacteriocin-producing *S. haemolyticus*

We screened overnight cultures from 174 *S. haemolyticus* isolates for bacteriocin inhibitory activity against three indicators: *Lactococcus lactis* IL1403 (60), a clinical *S. haemolyticus* 51-21 isolate (11, 19), and *Staphylococcus aureus* ATCC 25923. Colonies were picked from each of the 174 *S. haemolyticus* isolates from blood agar plates (Thermo Fisher Scientific, USA), then transferred to tryptic soy broth (TSB) (BD, USA/ Merck, Germany) and incubated with shaking at 37°C overnight.

We prepared 0.5 McFarland solutions in 0.85% saline of colonies from each of the indicator strains.

The suspensions were inoculated on Mueller Hinton (MH) agar (Oxoid, England) with a cotton swab and a rotator. Five microliters of overnight cultures, cell-free supernatant, or treated supernatant (heat, pH, protease) were spotted on the plates. Inhibition of bacterial growth was assessed visually after 20–24 hours. Three technical replicates were made of each plate. The genomes of *S. haemolyticus* isolates were submitted to the BAGEL4 webserver for identification of bacteriocin genes (20).

All except two *S. haemolyticus* isolates used in this study had been obtained and sequenced as part of previous studies (11, 19, 61). Of the isolates, 123 were of clinical origin, 46 were commensal isolates, and 4 were of veterinary origin. In addition, we tested a *S. haemolyticus*-type strain (CCUG 7323T) (62).

TABLE 3 Primer sets used for amplification of the bacteriocin cluster genes from *S. haemolyticus* 57-27 and plasmid pRMC2

Primer	Set	Sequence 5′–3′	Extension	Product
pRMC2_A1_FW	1	gtaccgttaggaggggttatttatgagtaaattagaactacttaatgaa	65°C, 6:00	7,786 bp
pRMC2_A1_RV		tgaattcgagctttatatgaataaactttctgagttggatgaaataag		
pRMC2_A1_vec_FW	2	cccctcctaacgctaccatcatgcttattttaattatactctatcaatgatag	3:30	6,439 bp
pRMC2_A1_vec_RV		tttattcatataaagctcgaattcactggc		
M2_INS_RV	3	gatgagatggaaggagatattattaatggaagtatagg	1:00	785 bp
pRMC2_INS_FW		gcctcttcgctattacgccag		
M1_INS_FW	4	ccttcattatgactatcaccttggtttaattctatag	1:00	1,084 bp
pRMC2_A1_vec_RV		ctgttaatcactttacttttatctaatctagacatcattaattc		

Heterologous expression of bacteriocin gene cluster

The genes required for bacteriocin core peptide production and those for modification, transport, and maturation were cloned into plasmid pRMC2 (Addgene, #68940) (Fig. S1). This plasmid allows anhydrous tetracycline-inducible expression of cloned genes (22).

We amplified the genes Lan A1-M2 (excluding Lan E-F) using primer set 1 (Table 3), following a two-step PCR protocol due to the AT-rich nature of the bacteriocin gene cluster sequence (63). We amplified the pRMC2 plasmid by PCR using primer set 2 (see Table 3 below). Both PCRs used Q5 High-Fidelity 2× Master Mix [New England Biolabs (NEB), USA]. Amplicons from both PCRs were digested with DpnI (NEB) before being cleaned up using the E.Z.N.A. Cycle Pure Kit (Omega, USA). We assembled the amplicons using NEBuilder HiFi DNA Assembly Master Mix (NEB) to form plasmid pRMC2_Romsacin. The newly assembled plasmids were transformed into NEB 5-alpha Competent *E. coli*, which we spread out onto Luria-Bertani (LB) + 100 µg/mL ampicillin and incubated overnight at 37°C. Correct assembly of the bacteriocin cluster in the plasmid was confirmed by colony PCR using primer sets 3 and 4 and OneTaq 2× MasterMix (NEB). We isolated the plasmids from *E. coli* using the NucleoSpin Plasmid Kit (Macherey Nagel, Germany) and concentrated them using Pellet Paint (Merck, USA).

We selected *S. aureus* RN4220 as a host for heterologous gene expression due to the ease with which it can be transformed, compared with other staphylococci. To make competent RN4220, we grew an overnight culture in 5 mL of TSB (37°C, shaking at 250 rpm) and diluted it with pre-warmed TSB to an optical density of 0.5 at 600 nm. The bacteria were returned to the incubator for 40 minutes before being harvested by centrifuging at 5,000 × *g* for 10 minutes. The pellet was washed in ice-cold sterile Milli-Q water before centrifuging at 5,000 × *g*. This step was repeated once. Following washing, we resuspended the cells in a 1:10 volume of ice-cold sterile 10% glycerol before centrifuging at 5,000 × *g* for 10 minutes. This step was repeated, but the volume of 10% glycerol was successively reduced each subsequent step to 1:25, 1:10, 1:100, and finally 1:200. Competent cells were aliquoted and frozen at −70°C until use.

Before electroporation, the competent cells were thawed on ice for 5 minutes and then on the bench for 5 minutes before being centrifuged at 5,000 × *g* for 1 minute. The supernatant was removed, and the cells were resuspended in sterile 10% glycerol with 0.5 M sucrose. We added 1 µg of plasmid to the cells and incubated them on the bench for 10 minutes. The cells were then transferred to a 1-mm electroporation cuvette (Biorad) and electroporated at 2.5 kV, 100 Ω, 25 µF (GenePulser Xcell, Biorad). We added 950 µL of TSB + 0.5 M sucrose (filter sterilized) to the cells and transferred them to a clean Eppendorf tube before incubating them for 1 hour at 37°C with shaking at 250 rpm. After recovery, we plated out 100-µL aliquots onto TSB + 10 µg/mL chloramphenicol before overnight incubation at 37°C. Presence of the plasmid was confirmed by PCR.

To induce the expression of the gene cluster, we added anhydrous tetracycline (0–2 µg/mL) to the TSB growth media of overnight cultures of RN4220 carrying pRMC2_Romsacin. We spotted 5 µL of cell-free supernatant (treated at 100°C before use) on plates of *L. lactis* IL 1403 indicator strain, as described in the previous section. As controls, we used wild-type RN4220 (no plasmid) and growth media with

anhydrous tetracycline (no bacteria). We used the *S. haemolyticus* bacteriocin producer for comparison of the results.

Bacteriocin stability

We exposed aliquots of concentrated cell-free supernatants to various treatments prior to antimicrobial testing, performed as described above. The aliquots were exposed to 4, 10, 20, 30, 40, 50, 80, 90, 100, or 121°C for 15 minutes. The pH was adjusted to 2.1, 8.6, 9.3, 10.5, and 11.9 with sodium hydroxide (NaOH) or hydrochloric acid (HCl) and incubated at room temperature for 30 minutes. We used trypsin (200 µg/mL) to test protease sensitivity. Concentrated cell-free supernatant was treated with the enzyme for 1.5 hour at 37°C.

Bacteriocin purification

Bacteriocin purification was performed similarly as described by Ovchinnikov et al. (56), with some modifications. One liter of BHI was inoculated with 2% (vol/vol) of an overnight culture of *S. haemolyticus* 57-27. The culture was incubated with vigorous shaking at 37°C for 24 hours, before cells were removed by centrifugation (10,000 × *g*, 4°C, 35 minutes). Proteins were then precipitated by the addition of 373-g ammonium sulphate per liter supernatant and left at 4°C overnight. Precipitated proteins were collected by centrifugation (12,000 × *g*, 4°C, 45 minutes). The protein pellet was dissolved in 200-mL Milli-Q water (Invitrogen, USA) and filtered through a 0.2-µm filter (Millipore, USA). The crude concentrate was freeze dried until use.

Freeze-dried concentrate precipitated from 1-L culture was dissolved in 200-mL Milli-Q water. The pH was adjusted to 4.5 (±0.5) and then applied on a HiPrep 16/10 SP-XL column (GE Healthcare, USA) equilibrated with Milli Q water (pH 4.5). The column was washed with 100 mL of 20 mM sodium phosphate buffer (pH 7) before elution of the bacteriocin with 100 mL of 0.5 M NaCl. The eluate was applied to a resource RPC column (1 mL) connected to an ÅKTA purifier system (GE Healthcare, USA). Water containing 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich, USA) was used as buffer A. We used a linear gradient of 2-propanol (Merck, USA) with 0.1% TFA (buffer B) for elution. The flow rate was 2–4 mL/min.

Antimicrobial activity in RPC purified fractions was determined quantitatively in 96-well plates using *L. lactis* 1403 as indicator strain. Briefly, overnight culture of *L. lactis* 1403 was diluted 50-fold in GM17 broth (Oxoid, England) in the wells of 96-well plates (Sarstedt, Germany) containing a serial dilution of the RPC fraction following incubation for 5–6 hours at 30°C. The growth was measured spectrophotometrically at 600 nm using SPECTROstarNano (BMG LABTECH, Germany). Purification was repeated so bacteriocin from 4 L of bacterial culture was purified all together. Fractions with bacteriocin activity were pooled.

MALDI-TOF mass spectrometry

MALDI-TOF MS was performed on an ultrafleXtreme mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflectron mode. The instrument was calibrated with peptide calibration standard II (Bruker Daltonics), and positive ions in the range 1,000 to 6,000 m/z were analyzed. The RPC purified fraction and matrix (HCCA; α-cyano-4-hydroxycinnamic acid) were mixed in equal volumes and spotted on a Bruker MTP 384 steel target plate (Bruker Daltonics) for analysis.

Bacteriocin inhibition

The activity of the purified fractions was tested against WHO priority pathogens and a broad range of Gram-positive indicators with agar spot-on-lawn assay and planktonic growth inhibition (Table S1; Table 2).

We used a similar method as described by Holo (64) for the spot-on-lawn assy. Briefly, we made a 50-fold dilution of overnight culture of indicator strains in 5-mL BHI soft agar and plated out as a lawn on BHI agar plates (BD, USA). Afterwards, we spotted 3 µL of the bacteriocin on the lawn and incubated at 30°C for 24 hours. Inhibition of bacterial growth appeared as clear zones.

We performed planktonic growth inhibition by following the colony suspension (3A) and broth microdilution for antimicrobial peptides (4E) methods in the Wiegand protocol (65). The starting concentration of the bacteriocin in the MIC assay was a 1/10 or 1/5 dilution of the purified bacteriocin in water. We used 96-well plates (Falcon, USA) and MH broth (BD, USA) for the dilution series and performed three technical replicates. We report the dilution factor resulting in 50% inhibition of the indicator strain.

Biofilm confocal microscopy

We assessed the bacteriocin effect on biofilm-associated *S. haemolyticus* (nos. 1 and 6), *S. epidermidis* (nos. 4 and 6), MRSA (no. 1), and VRE (no. 2) cells by confocal microscopy. Biofilms were established in four-well cover glass slides (Thermo Fisher Scientific, USA). Overnight cultures were diluted 1:10 in TSB with 1% glucose, and 500 µL was transferred to each well in the glass slides. Staphylococcal biofilms grew 24 hours and *E. faecium* for 48 hours at 37°C before the wells were washed twice with PBS (Sigma-Aldrich, USA). We dissolved and diluted the purified bacteriocin 1/2 in TSB with 1% glucose before addition to the biofilm. Five hundred microliters of bacteriocin or control (TSB with 1% glucose) were added to the wells and incubated for 24 hours at 37°C. Wells were carefully washed twice with PBS and stained for 20 minutes with LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, US) (1-µL dye per milliliter PBS). Dye was removed, and 500-µL PBS was added to each well.

For confocal microscopy, we used a Zeiss LSM780 equipped with a 10×/0.45 M27 Plan Apochromat objective with digital zoom and ZEN v.2.3 software (ZEISS, Germany). We used the SmartSetup function in ZEN to adjust the channels. Pictures are 212.55 × 212.55 µm, with a pixel size of 255 nm. We took pictures from representative areas in the chamber wells. All photos are taken using the same settings.

Bacteriocin units

The appropriate BU concentrations for the propidium iodide pore formation assay and scanning electron microscopy were determined by a microtiter plate assay. Briefly, twofold dilutions of purified romsacin, micrococcin P1, and nisin A in M17 medium supplemented with 0.5% glucose (GM17) were prepared in the wells of a microtiter plate to a volume of 100 µL per well. Each well was inoculated with 100 µL of a 25-fold diluted overnight culture of *L. lactis* IL1403 (50-fold final dilution). A bacteriocin unit was defined as the amount of bacteriocin that inhibited the indicator strain by at least 50% in 200-µL culture compared to the turbidity of a positive control with no added antimicrobial. Turbidity was measured spectrophotometrically at 600 nm using a SPECTROStar Nano microplate reader (BMG LABTECH, Germany).

Propidium iodide pore formation assay

An overnight culture of the indicator strain *L. lactis* IL1403 was washed twice in PBS (5,000 × *g*, 5 minutes), and resuspended to an OD_{600} of 3. We used a black microtiter plate to dilute romsacin, nisin A, and micrococcin P1 to 50 BU/mL in 100 µL of PBS containing 40 µM propidium iodide (see section above for bacteriocin units; BU). We added 100 µL of indicator to a final OD of 1.5 to each well containing diluted antimicrobial substance. Fluorescence was kinetically measured every 10 minutes for 3 hours with excitation at 535/20 nm (515–555 nm) and emission at 630/40 nm (590–670 nm) using a Hidex Sense microplate reader (Hidex, Finland).

Scanning electron microscopy

L. lactis IL1403 was grown to mid-log phase (OD_{600} ~0.5) and incubated with 50 BU/mL of romsacin for 30 minutes at 30°C (see section above for bacteriocin units). We used a culture with no bacteriocin added as control. After incubation, cells were harvested by centrifugation at 10,000 × *g* for 5 minutes, washed twice in PBS, and resuspended in fixing solution (1.25%, wt/vol, glutaraldehyde, 2%, wt/vol, formaldehyde, PBS) for overnight incubation at 4°C. Fixed cells were then washed three times in PBS and allowed to sediment/attach to poly-L-lysine-coated glass coverslips at 4°C for 1 hour. Attached cells were dehydrated with an increasing ethanol series (30%, 50%, 70%, 90%, and 96%, vol/vol) for 10 minutes each and finally washed four times in 100% ethanol. Cells were dried by critical-point drying using a CPD 030 critical point dryer (BAL-TEC, USA). Coverslips were sputter coated with palladium-gold using a Polaron Range sputter coater (Quorum Technologies, UK). Microscopy was performed on an EVO 50 EP scanning electron microscope (Zeiss, Germany) at 20 kV and a probe current of 15 pA. The SEM analysis was performed twice independently.

Preparations for SEM analysis of MRSA (no. 1), *S. haemolyticus* (no. 1), *S. epidermidis* (no. 6), and *B. subtilis* 168 were done in the same manner as for *L. lactis*, but with some exceptions. We used a Leica EM CPD 300 critical point dryer (Leica, Germany), a Polaron sputter coater SC7640 (Quorum Technologies, USA), and a Gemini SEM 300 scanning electron microscope (Zeiss, Germany). We used romsacin concentrations above MIC for the respective strains in the SEM assay.

Growth curve

We investigated the bacteriostatic or bacteriolytic potential of romsacin by making growth curves of MRSA (no. 1) and *S. haemolyticus* (no. 1). Overnight cultures in MH broth were diluted 1:50 in fresh media and grown to OD_{600} 0.5. A pellet of romsacin was dissolved in MH broth and mixed 1:1 with the bacterial culture. Bacterial culture mixed with 1:1 with MH broth was used as control. A 96-well microplate was incubated in Synergy H1 (Bio-Tek, USA) at 37°C for 21 hours, and the turbidity of the solutions was read at OD_{600} every 10 minutes. We made a CFU count at 0 and 21 hours.

Membrane integrity assay

We investigated the membrane disruptive properties of romsacin by using a bioluminescence-based assay described by Virta et al. (66). The method measured membrane permeabilization with D-luciferin as a substrate. D-luciferin hardly crosses biological membranes at neutral pH, but membranolytic agents allow it to enter the cell and emit light.

The test strain was *B. subtilis* 168 carrying plasmid pCSS962, which expresses luciferase and emits luminescence if externally added D-luciferin enters the bacterial cells after membrane disruption. We used chlorhexidine (200 µg/mL) as a reference. Chlorhexidine is known for its membrane disruption properties (25). *B. subtilis* 168 were grown overnight in MH medium with 5 µg/mL chloramphenicol. A dilution of the overnight culture was made in MH medium without antibiotics, and the culture was grown for around 4 hours. Undiluted antimicrobial compounds and six dilutions were used (1/2 to 1/64), and water, as control. Five microliters of the antimicrobial dilution series and water were mixed with 95 µL of an over-day culture of *B. subtilis* in black round-bottom 96-well plates (Nunc, Denmark). Plates were read immediately in a Synergy H1 reader (BioTek, USA). Monitoring of luminescence was done from 0 to 4 minutes after addition of the antimicrobial compound.

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DATA AVAILABILITY

The whole-genome sequencing assembly for isolate *S. haemolyticus* 57-27 is available in the European Nucleotide Archive at the accession number GCA_903969855.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplementary Figure 1 (Spectrum00869-23-s0001.pdf). Plasmid pRMC2.
Supplementary Table 1 (Spectrum00869-23-s0002.xlsx). Romsacin inhibition.

Open Peer Review

PEER REVIEW HISTORY (review-history.pdf). An accounting of the reviewer comments and feedback.

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