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Bacteriocin-based treatment of *Staphylococcus haemolyticus* biofilm

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Biotechnology

Acknowledgements

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Mikkel Brønner

Abstract

Antibiotics have long been used to treat infections caused by pathogenic and multidrug-resistant (MDR) bacteria. However, an increasing number of bacteria, including pathogenic bacteria, have developed resistance against most antibiotics. As a consequence, there is now a great need for the development of novel antimicrobial agents that can be utilized in clinical settings. One potential option is a subgroup of antimicrobial peptides known as bacteriocins, which have many advantageous properties which make them viable and very attractive alternatives to conventional antibiotics in the fight against pathogenic bacteria. In this study, the newly designed and bioengineered hybrid bacteriocin EntK1EJ97, which interacts with the Zn-dependent RseP receptor and causes pore formation in the bacterial cell membrane, revealed a relatively broad-spectrum of activity against a sizable collection of Gram-positive bacterial species from various genera and was very active against the clinically important and biofilm producing opportunistic pathogen *Staphylococcus haemolyticus*. The bacterial species *S. haemoyticus* is a member of the coagulase-negative staphylococci (CoNS) and may cause both local and systemic infections in humans. Comparative antimicrobial susceptibility with conventional antibiotics and EntK1EJ97 showed that EntK1EJ97 could be an attractive alternative treatment against *S. haemolyticus*, although EntK1EJ97-resistant mutants was generated. Microtiter plate biofilm assay with crystal violet staining of biofilm producing sessile cells revealed that *S. haemolyticus* was a very strong *in vitro* biofilm producer under optimal conditions. For the purpose of preventing the generation of planktonic and sessile biofilm producing EntK1EJ97-resistant mutants, various single and combinatorial bacteriocin treatments were applied *in vitro* in which the triple combinatorial EntK1EJ97/GarKS/MP1 treatment was most effective. The EntK1EJ97-resistant phenotype was investigated through temperature-stress assay, sequencing of the *rseP* gene encoding the RseP receptor, and by evaluating any morphological differences with the use of TEM. The temperature-stress assay and sequencing indicated that the RseP was functional and that no mutations had occurred at the DNA-level of the RseP. However, interesting differences in the thickness and structure of the EntK1EJ97-sensitive and resistant mutants cell wall was observed which might perhaps explain the phenotype of the EntK1EJ97-resistant *S. haemolyticus* mutants.

Sammendrag

Antibiotika har lenge vært brukt til å behandle infeksjoner forårsaket av patogene og multiresistente (MDR) bakterier. Imidlertid har et økende antall bakterier, inkludert patogene bakterier, utviklet resistens mot de fleste antibiotika. Som en konsekvens er det nå et stort behov for utvikling av nye antimikrobielle midler som kan brukes i kliniske omgivelser. Et potensielt alternativ er en undergruppe av antimikrobielle peptider, kjent som bakteriociner, som har mange fordelaktige egenskaper som gjør dem levedyktige og veldig attraktive alternativer til konvensjonelle antibiotika i kampen mot sykdomsfremkallende bakterier. I denne studien avslørte den nylige designede og bioingenierte hybride bakteriocinet EntK1EJ97, som interagerer med den Zn-avhengige RseP-reseptoren og forårsaker poredannelse i bakteriecellemembranen, et relativt bredt spekter av aktivitet mot en betydelig samling av Gram-positive bakteriearter fra forskjellige slekter og var veldig aktiv mot den klinisk viktige og biofilmproduserende opportunistiske patogenen *Staphylococcus haemolyticus*. Den bakterielle arten *S. haemoyticus* er medlem av de koagulase-negative stafylokokkene (CoNS) og kan forårsake både lokale og systemiske infeksjoner hos mennesker. Sammenlignende antimikrobiell mottakelighet med konvensjonelle antibiotika og EntK1EJ97 viste at EntK1EJ97 kan være en attraktiv alternativ behandling mot *S. haemolyticus*, selv om EntK1EJ97-resistente mutanter ble generert. Mikrotiterplate-biofilmanalyse med krystallfiolett farging av biofilm-produserende sessile celler avslørte at *S. haemolyticus* var en veldig sterk *in vitro* biofilmprodusent under optimale forhold. For å forhindre generering av planktoniske og sessile biofilm-produserende EntK1EJ97-resistente mutanter, ble forskjellige enkelt- og kombinatoriske bakteriocinbehandlinger brukt *in vitro* der den tredobbelte kombinatoriske EntK1EJ97/GarKS/MP1-behandlingen var mest effektiv. Den EntK1EJ97-resistente fenotypen ble undersøkt gjennom temperatur-stressanalyse, sekvensering av *rseP*-genet som koder for RseP-reseptoren, og ved å evaluere eventuelle morfologiske forskjeller ved bruk av TEM. Temperatur-stressanalysen og sekvenseringen indikerte at RseP var funksjonelt og at ingen mutasjoner hadde skjedd på DNA-nivået til RseP. Imidlertid ble det observert interessante forskjeller i tykkelsen og strukturen til celleveggen hos EntK1EJ97-sensitive og resistente mutanter, noe som kanskje kan forklare fenotypen til de EntK1EJ97-resistente *S. haemolyticus* mutantene.

List of abbreviations

ATP – Adenosine triphosphate

BHI – Brain heart infusion

BLAST – Basic local alignment search tool

BOAT – Biofilm-oriented antimicrobial test

bp – Base pair

CaCo – Sodium cacodylate

CoNS – Coagulase-negative staphylococci

dH_2O – Distilled water

DNA – Deoxyribonucleic acid

EMIC – Equivalent minimum inhibitory concentration

EPS – Extracellular polymeric substance

Eq – Equivalent concentration

FRET – Fluorescence resonance energy transfer

GLU – Glucose

LAB – Lactic acid bacteria

LMG – Laboratory of Microbial Gene-technology

LTA – Lipoteichoic acid

MDR – Multidrug resistance

MIC – Minimum inhibitory concentration

mRNA – Messenger RNA

MRSA – Methicillin-resistant *Staphylococcus aureus*

MSA – Multiple sequence alignment

MSCRAMMs – Microbial surface components recognizing adhesive matrix molecules

Mut – Mutant

NaCl – Sodium chloride

NCBI – National Center for Biotechnology Information

NMR – Nuclear magnetic resonance

OD – Optical density

O/n – Overnight

PCR – Polymerase chain reaction

PSA – Pairwise sequence alignment

RNA – Ribonucleic acid

RPC – Reverse-phase chromatography

RT – Room temperature

SEM – Scanning electron microscopy

TEM – Transmission electron microscopy

TFA – Trifluoroacetic acid

TSB – Tryptic soy broth

TTC – 2,3,5-triphenyltetrazolium chloride

WHO – World Health Organization

Wt – Wildtype

WTA – Wall teichoic acid

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1. Introduction

1.1. Antibiotics and the issue with resistant pathogenic bacteria

Antibiotics are secondary antibacterial metabolites produced by microorganisms; a variety of bacteria and fungi produce these compounds to inhibit or kill competing microorganisms. Today, thousands of different antibiotics are known, but less than 1% of them are clinically useful. Undoubtedly, the discovery of antibiotics is one of the greatest revolutions in chemotherapy and medicine in recent times. The huge impact that the use of antibiotics had on medicine turned them into “miracle drugs” which the medical world was craving for.

However, with the intensive use of antibiotics in clinical settings, veterinary and in the agriculture an increasing number of bacteria, including pathogenic bacteria, have developed resistance against their antimicrobial effects [89]. The history of antibiotic-resistant bacteria is as old as antibiotics themselves, and there are many theories on how and why antibiotics arose. Some of today’s scientists within this field believe that evolution of genetic elements encoding antibiotics and genes making the microbes intrinsically resistant against the antibiotics they produce is the result of random genetic mutations. These random genetic mutations gave the microorganisms carrying the mutation a competitive advantage in environments with limited macronutrients (such as carbon, oxygen, hydrogen, nitrogen, sulfur, phosphorus, potassium, calcium, magnesium, and iron) and micronutrients (such as manganese, zinc, cobalt, molybdenum, nickel, and copper). Eventually, other bacterial species with different genetic mutations arose, some of which also led to resistance against these antibiotics. This in turn led to a selection of these resistant bacteria rather than bacteria without this genetic mutation [25].

As seen in **Figure 1.1**, it was becoming harder to identify new naturally-occurring effective antibiotics by the mid-1960s, and an ever-increasing resistance to already established antibiotics was observed in clinically-relevant pathogens. These issues spawned the era of medicinal chemistry, the next period of innovation in the antibiotic discovery; where the focus was largely on creating synthetic versions of the natural occurring antibiotics. These derivatives led to outstanding improvements in the application of antibiotics, which included lower doses, expanded antimicrobial spectrum and the bypass of bacterial resistance

mechanisms, at least in the first stages of their clinical use [14]. However, we are now entering an era where most of the antibiotics that could be easily developed using this method have been acquired.

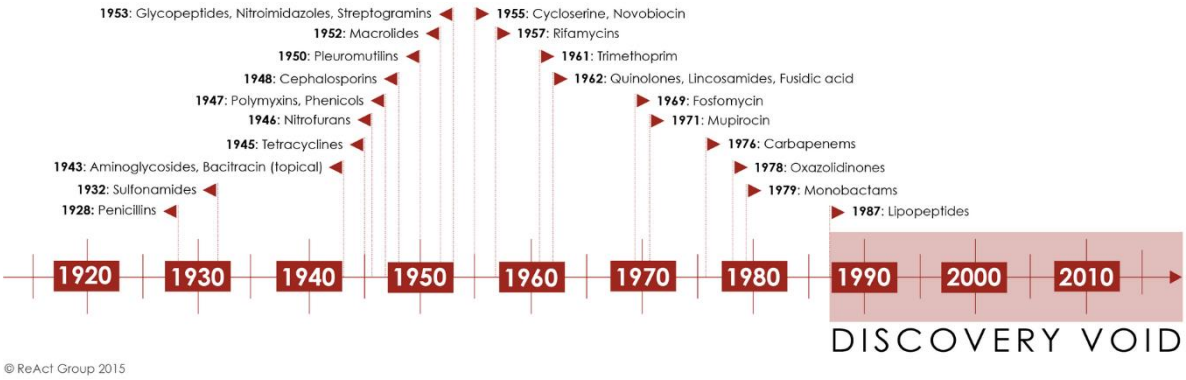

Figure 1.1. History of major antibiotic discovery. "The discovery void" refers to the period from 1987 until today, as the last antibiotic class that has been successfully introduced as treatment was discovered in 1987. The figure is adapted from [99].

Antibiotics are being utilized worldwide in several different fields including the food production chain, agriculture, and the health sector. They have become an important remedy in the fight against disease and infections, and the greatest daily effect we as individuals experience of antibiotics is their contribution to improving human health [76]. Antibiotics save lives, so there is no doubt that antibiotic-resistance is a problem for every individual, society and the world in general. It has been estimated that around 100 000 – 200 000 metric tons of various antibiotics are being produced and utilized annually worldwide. The overuse of antibiotics greatly contributes to the increased antibiotic-resistance that we see at the present time.

As seen in **Table 1.1**, there are many different types of antibiotics known that each have their specific mode of action [70]. They are generally classified according to the bacterial structures or processes in which they interact with, as well as their chemical structure. These can be divided into two main categories; synthetic antibiotics and natural occurring antibiotics, where these are again subdivided further into different subgroups of antibiotics. Antibiotics and other antimicrobial agents can also be classified according to whether their effect on the bacteria is deadly or merely hinders growth and reproduction. Antibiotics and

other antibacterial agents that kill bacteria are known as bactericides, while the substances that affect their growth and reproduction are known as bacteriostatic compounds [70].

Table 1.1. The different classes of antibiotics including their distinctive features and mode of action.

Class of antibiotics	***Distinctive feature***	***Mode of action***	***Examples***
β-lactams	*All contain a beta-lactam ring*	*Inhibit bacterial cell wall biosynthesis*	*Penicillins such as amoxicillin and flucloxacillin; Cephalosporins such as cefalexin*
Aminoglycosides	*All contain aminosugar substructures*	*Inhibit the synthesis of proteins by bacteria, leading to cell death*	*Streptomyci, neomycin, kanamycin, paromomycin*
Chloramphenicol	*Distinct individual compound*	*Inhibit synthesis of proteins, preventing growth*	
Glycopeptides	*Consist of carbohydrate linked to a peptide formed of amino acids*	*Inhibit bacteria cell wall biosynthesis*	*Vancomycin, taicoplanin*
Quinolones	*All contain fused aromatic rings with a carboxylic acid group attached*	*Interfere with bacteria DNA replication and transcription*	*Ciprofloxacin, levofloxacin, trovafloxacin*
Oxazolidinones	*All contain 2-oxazolidone somewhere in their structure*	*Inhibit synthesis of proteins by bacteria, preventing growth*	*Linezolid, posizolid, tedizolid, cycloserine*
Sulfonamides	*All contain the sulfonamide group*	*Do not kill bacteria but prevent their growth and multiplication.*	*Prontosil, sulfanilamide, sulfadiazine, sulfisoxazole*
Tetracyclines	*All contain 4 adjacent cyclic hydrocarbon rings*	*Inhibit synthesis of proteins by bacteria, preventing growth*	*Tetracycline, doxycycline, lymecycline, oxytetracycline*
Macrolides	*All contain a 14-, 15-, or 16-membered macrolide ring*	*Inhibit protein synthesis by bacteria, occasionally leading to cell death*	*Erythromycin, clarithromycin, azithromycin*
Ansamycins	*All contain an aromatic ring bridged by an aliphatic chain*	*Inhibit the synthesis of RNA by bacteria, leading to cell death*	*Geldanamycin, rifamycin, naphthomycin*
Streptogramins	*Combination of two structurally different compounds from groups denoted A & B*	*Inhibit the synthesis of proteins by bacteria, leading to cell death*	*Pristinamycin IIA, pristinamycin IA*
Lipopeptides	*All contain a lipid bonded to a peptide*	*Disrupt multiple cell membrane functions, leading to cell death*	*Daptomycin, surfactin*

Antibiotics and other antimicrobial agents generally attack bacterial cells, not the host organism's cells. This is due to the fact that bacterial cells and the host organism's cells have different cellular structures and different metabolic pathways. This allows antibiotics and other antimicrobial agents to have a selective toxicity towards bacterial cells [9]. The susceptibility of some bacteria to individual antibiotics varies considerably, for instance, Gram-positive bacteria and Gram-negative bacteria differ in their sensitivity towards different types of antibiotics. An example of this is that Penicillin G generally affects Gram-positive bacteria, while most Gram-negative bacteria are naturally resistant to this type of antibiotic. Certain broad-spectrum antibiotics, such as tetracycline, are generally effective against both groups [70]. As seen in **Figure 1.2**, typical bacterial targets for antibiotics includes cell wall synthesis, disruption of cell membrane integrity and function, inhibition of both the 50S and 30S subunits forming a functional bacterial ribosome, various metabolic pathways and the enzymes involved in them and inhibition of nucleic acid synthesis so that replication and transcription do not occur, are some of the major effects these drugs have on bacteria [9].

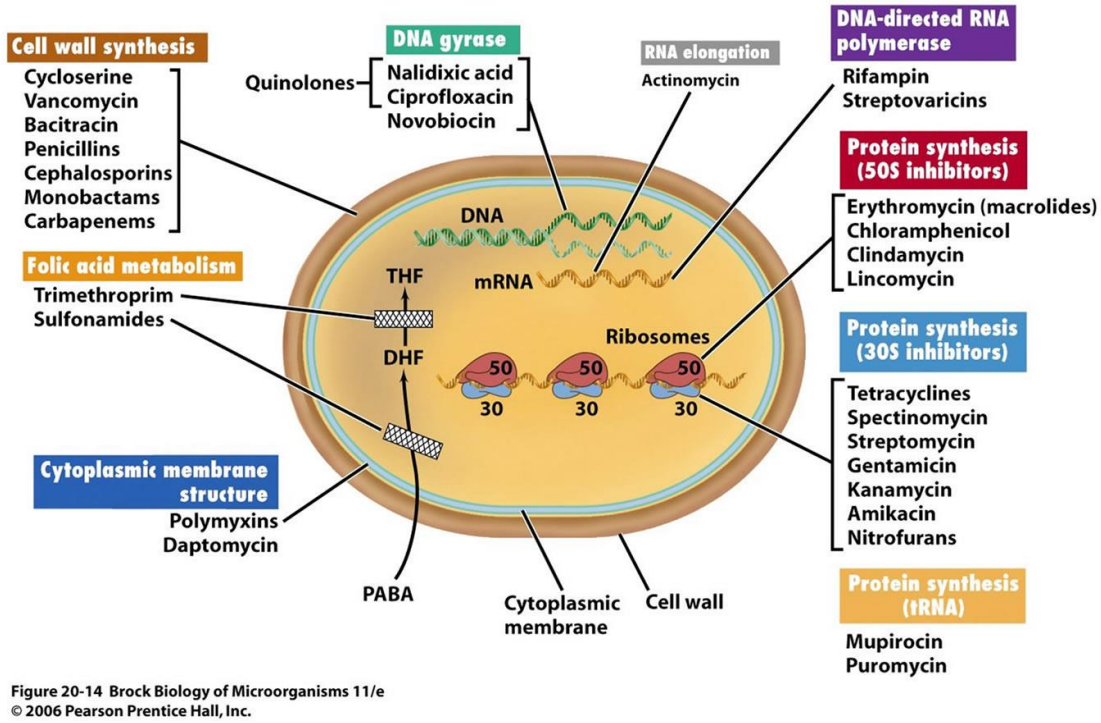

Figure 1.2. Bacterial targets of major antimicrobial agents. Agents are classified according to their target structures in the bacterial cell. THF = tetrahydrofolate; DHF = dihydrofolate; mRNA = messenger RNA. The figure is adapted from [70].

Antimicrobial resistance is a growing problem worldwide, and in 2014 the World Health Organization (WHO) proclaimed the expanding issue of antibiotic resistant pathogens as one of the largest challenges human society will face in the coming decades [114]. In 2015, it was estimated that approximately 33 000 people die annually just in Europe alone, as a direct consequence of pathogenic bacteria resistant towards antibiotics [18]. Antimicrobial resistance is defined as the innate or acquired property of a microorganism to be resistant or insensitive to the effect of one or more antimicrobial agents [70], and mechanisms of resistance is broadly divided into three categories: i) inactivation of the antimicrobial agent, ii) removal of the antimicrobial agent from the bacterial cell, and iii) modification of the anti-bacterial target in such degree that the antimicrobial agent no longer binds with high enough affinity to be effective [112].

Genes encoding antimicrobial resistance are present in any organism that produces the specific antibiotic agent, where these genes can be transmitted between bacteria by lateral or horizontal gene transfer. Lateral gene transfer occurs between microorganisms of the same species, while horizontal gene transfer occurs between microorganisms of different species. As seen in **Figure 1.3**, Conjugation, transduction and transformation are the mechanisms by which bacteria transfer genetic material between themselves. Conjugation is a mechanism for transmission of genetic material, where the donor and acceptor bacteria are dependent on cell-cell contact to transfer the genetic material. In transduction, the genetic material is transmitted by bacteriophages (bacterial viruses) that only infect bacteria and transmits genetic material that it has picked up from another bacteria, whereas transformation allows bacteria to take up free DNA located in their extracellular environment [70].

Antibiotic resistance among microorganisms can be genetically encoded on their circular chromosome or, in most cases, on a plasmid. These plasmids are often referred to as R-plasmids (“R” for resistance) and most of the genes encoding resistance are located on these horizontally transmitted plasmids. These genes may encode enzymes that modify and inactivate the antimicrobial agent, genes that encode enzymes that inhibit the uptake of the antibiotic agent, or that encode enzymes that actively pumps them out of the cell such as efflux-pumps [70]. These plasmids may contain several different genes for resistance and duplicated plasmids containing these genes can be transmitted to bacteria previously

sensitive to the antimicrobial agent, thus rendering this bacterium resistant or insensitive towards the particular antimicrobial agent.

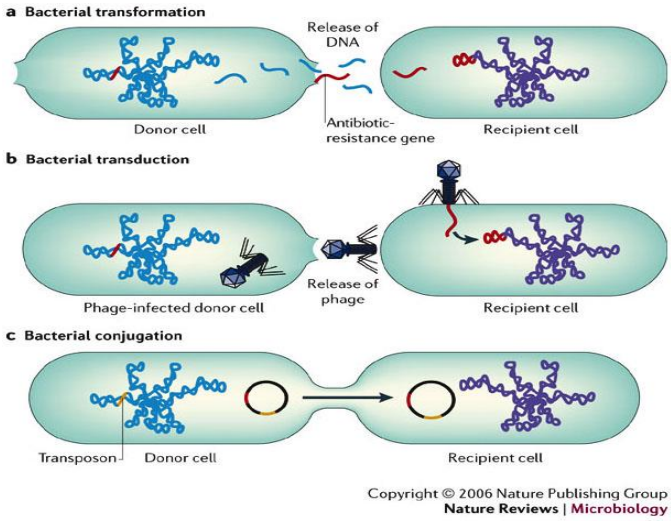

Figure 1.3. Mechanisms of horizontal gene transfer between bacteria. The figure is adapted from (Furuya and Lowy (2006), Nat Rev Microbiol. 4: 36-45. http://dx.doi.org/10.1038/nrmicro1325).

Bacteria have the ability to develop resistance towards any antimicrobial agents that relies solely upon a bacteriostatic or bactericidal mechanisms of action, and resistance towards a new antibiotic in clinical settings can develop within a period of just months to years after being introduced into clinical settings [70]. A relatively recent database has shown the existence of more than 20 000 potential resistance genes that emerged from nearly 400 different types of sequences of different available bacterial genomes. This is a clear danger signal, and scientists are now warning of a fallback to the pre-antibiotic era if this resistance development continues. In addition, long and costly production time as well as low prices no longer makes antibiotics a profitable investment for pharmaceutical companies. Thus, alternative approaches to antibiotics in the fight against resistant pathogenic bacteria are in great need [34].

1.2. Bacteriocins as an alternative treatment to conventional antibiotics

As a consequence of the increasing antibiotic-resistance seen in pathogenic bacteria, there is now a great need for the development of new antimicrobial agents that can be utilized in clinical settings. One of such potential options is a subgroup of antimicrobial peptides known as bacteriocins. Contrary to conventional antibiotics, which are synthesized through complex multi-enzyme pathways, bacteriocins are ribosomally synthesized antimicrobial peptides [26][63] that are active against other bacteria and against which the producer has a specific immunity mechanism system [30]. The antimicrobial inhibition spectra and potency (strength) of bacteriocins differ greatly with the majority displaying a narrow spectrum of activity, and are normally most active against closely related bacteria that are likely to occur in the same ecological niche, but some are also known to display a broad spectrum of activity and targets bacteria from several different genera [78]. Bacteriocins are synthesized as precursors on the bacterial ribosome and the mature peptides usually consist of 20 – 60 amino acid residues. The variation among these peptides are quite large in terms of length, amino acid sequence and composition, hydrophobicity, secretion and processing machinery and post-translational modifications; all of which ultimately influence their spectrum of antimicrobial activity [64][79][77][72].

Bacteriocins are most commonly classified based on their mechanism of action, mode of synthesis, structure and size. For example, bacteriocins produced by the Gram-positive lactic acid bacteria (LAB) are now classified in three different classes, as seen in **Figure 1.4**. Class I comprises small peptides with a high degree of post-transcriptional modification, and as such they are divided in subclasses according to what modified amino acids they contain. Class II consists of small and heat stable unmodified peptides, subdivided according to several criteria such as size, shape and whether it consists of one or more peptides. Class III is made up of larger and heat liable peptides, subdivided based on their ability to cause cellular lysis or not [7].

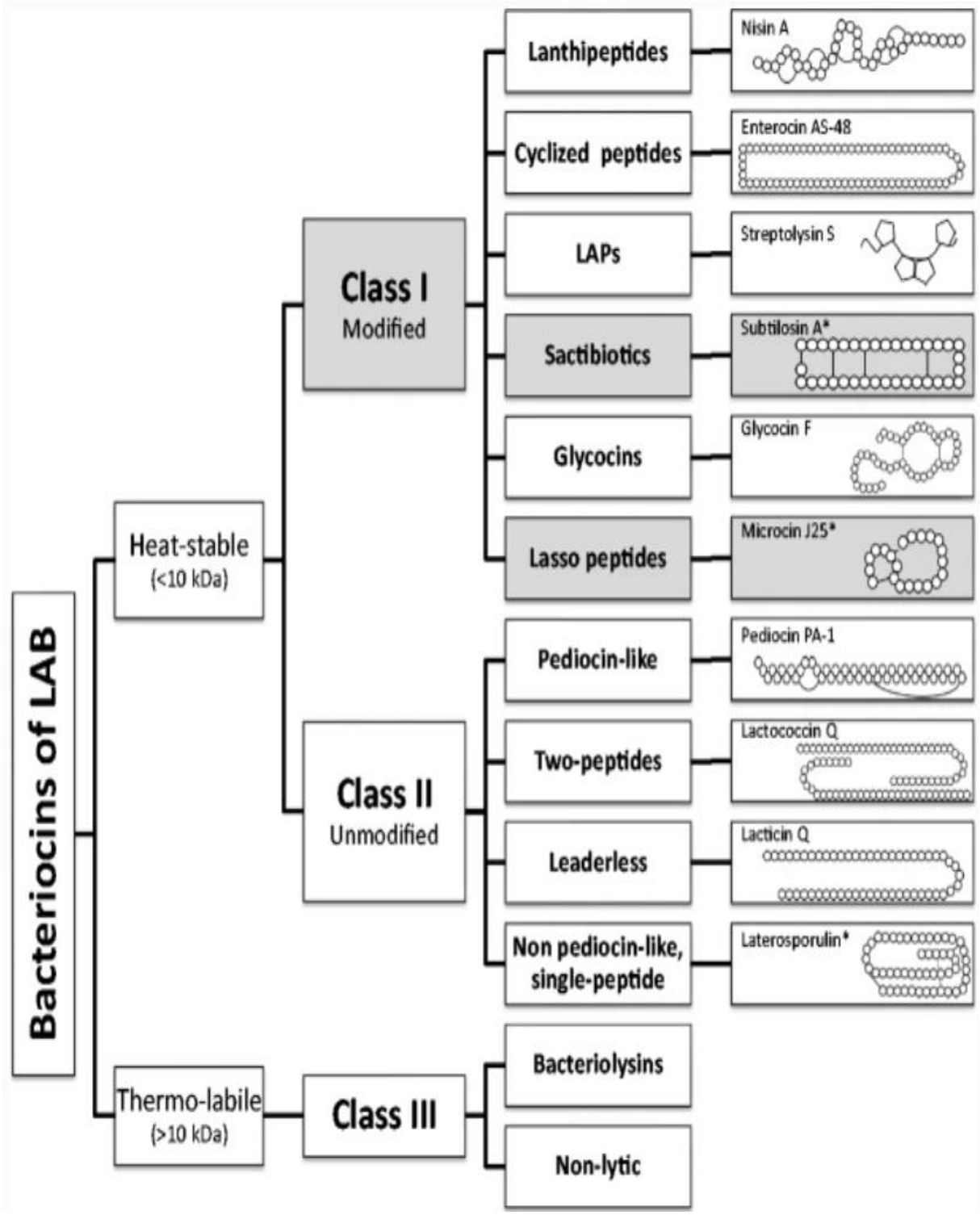

Figure 1.4. Proposed classification scheme for bacteriocins and their structures. Classes identified in silico are depicted in gray. Structure of non-lytic bacteriocins of class III still remains uncharacterized. *Bacteriocins from non-lactic acid bacteria. The figure is adapted from [7].

Many of these bacteriocins have a high specific activity against clinically relevant pathogens, including antibiotic-resistant strains. Importantly, bacteriocins have mechanisms of action that are distinct from those of the antibiotics and, given their protein nature, are amenable to genetically-based peptide bioengineering [31]. As opposed to conventional antibiotics, which often function as enzyme inhibitors, bacteriocins mechanism of action is that they generally create pores in the cell membrane of their target cells by either inserting themselves within the cell membrane or by interacting with specific membrane-associated receptors, as seen in **Figure 1.5**. This has critical effects on the affected bacterial cell leading to loss of cell membrane integrity, dissipation of proton motive force, ATP depletion and leakage of nutrients and metabolites out of the cell, thus killing the bacterial cell [24][15][1].

In order to disrupt the cell membrane and form pores, bacteriocins must interact with the cell membrane of the target cells and insert themselves into the membrane. This process is partly facilitated by electrostatic interactions between the positively charged peptide and anionic lipids that are abundantly present in the Gram-positive bacterial cell membranes [17]. This is also a process that is influenced by factors such as the membrane potential in the target cell and the pH in the environment, this means that a bacterial cells sensitivity towards a bacteriocin depends to some extent on the physiological state of the bacterial cell [42].

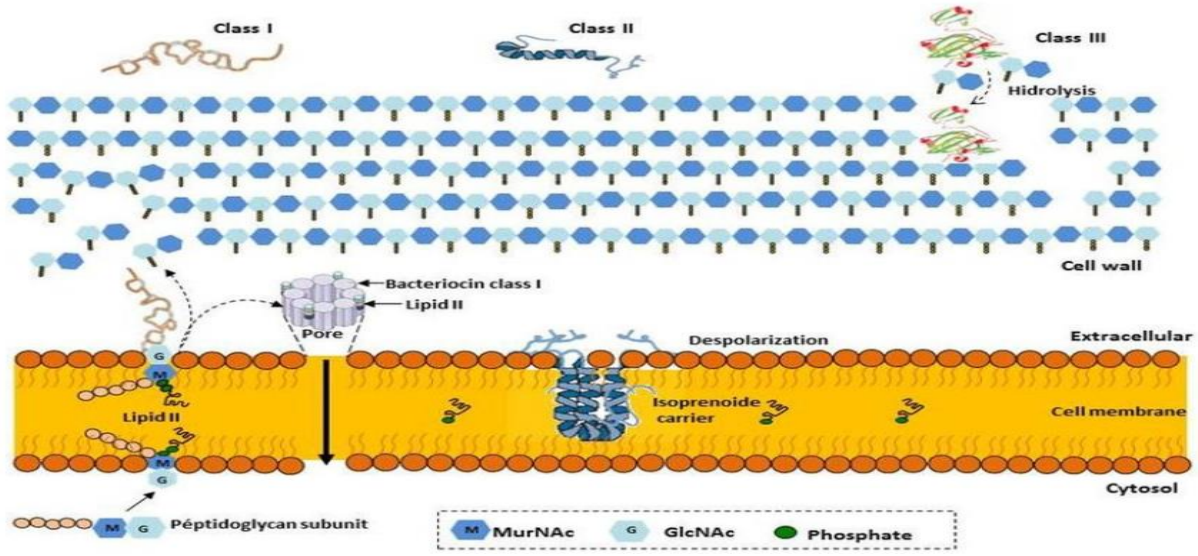

Figure 1.5. LAB bacteriocin class I, II and III mechanism of action. GlcNac = N-acetylglucosamine; MurNac = N-acetylmuramic acid. The figure is adapted from [31][6].

Bacteriocins have also been known to interact with specific receptors in the target cell membrane which leads to disruption of proper cell membrane function and eventually leading to cell death by its affinity and binding to the receptor. The presence of these membrane receptors has been supported by several functional studies where class II bacteriocins were only active against whole cells or vesicles containing cellular proteins, but not against protein-free vesicles, suggesting the presence of a receptor in the cell membrane with which it interacts [42]. These receptors have functions that are important to the cells survivability, for example involvement in key processes in the cells metabolism such as the mannose phosphotransferase system (Man-PTS) which is a four-domain membrane complex involved in the uptake of sugars in bacteria. Two of these domains, IIC and IID, are involved in the binding of several bacteriocins, such as the lactococcins, among others [38]. In addition, they have also been found to exert intracellular inhibitory activity by disrupting essential biological processes such as protein biosynthesis, cell division, biosynthesis of the cell wall and nucleic acid metabolism [38][29][10].

Although several broad-spectrum bacteriocins exist that can be used to target infections of unknown pathogens, potent narrow-spectrum bacteriocins have also been identified that can control targeted pathogens without negatively affecting commensal populations of bacteria [93][13]. Although these antimicrobial peptides show efficiency in killing pathogenic bacteria, the emergence of resistant bacteria is very much still a possibility, although it might be minimized through a detailed understanding of bacteriocin mechanisms of action and through peptide bioengineering [31].

Bacteriocins have many advantageous properties which makes them viable and very attractive alternatives to antibiotics in the fight against pathogenic bacteria. These properties includes their potency (as determined *in vitro* and *in vivo*), their low toxicity, the availability of both broad- and narrow-spectrum peptides, the possibility of *in situ* production by probiotics and the fact that these peptides can be bioengineered to increase their spectrum of activity and their potency [31]. In addition, bacteriocins display strong activity against target bacteria at pico- and nanomolar concentrations, making them even more potent than some antibiotics. Because of their peptide nature and because they are directly encoded by genes, bacteriocins are often more amenable to bioengineering than classical antibiotics. Bioengineering of bacteriocins can be carried out by bacteriocin gene

manipulation, can involve *in vitro* harnessing of the biosynthetic enzymes required for peptide production and/or can rely on partial or complete chemical synthesis of the antimicrobial. Bioengineering of these peptides have been important for furthering the understanding of the fundamentals of bacteriocin activity and structure-function relationships. There is also an increasing number of bioengineered peptides that exhibit enhanced functionalities such as activity and/or stability, which makes them more attractive from a clinical perspective. Bacteriocins are known to work through many distinct mechanisms of action that differ from those of antibiotics. These mechanisms can be broadly divided into those that function primarily at the cell membrane and those that are active primarily within the cell, affecting gene expression and protein synthesis [31].

Another advantage of bacteriocins is that they are generally less likely to induce resistance as most of them have several different targets and rarely interact with a specific receptor and among those that act on a single target, most act on the cell membrane where resistance development is more unlikely to occur [97]. However, as this study shows, the potential emergence of resistant pathogens is an issue for any antimicrobial to be used in clinical setting and one that must be addressed.

1.3. The bacteriocins used in this study

The antimicrobial activity of a handful different bacteriocins was investigated during this study. Special attention was given to the non-modified antimicrobial peptides of the LsbB-like bacteriocin family in class IId leaderless bacteriocins. The LsbB-like bacteriocin family presently contains four sequence related members which includes LsbB, EntK1, EntQ and EntEJ97. As seen in **Figure 1.6**, these peptides have a quite conserved C-terminal region which contains a characteristic KXXXGXXPWE motif, this might indicate the same receptor binding site for these bacteriocins and that the C-terminal part is responsible for the receptor interaction. Their mechanism of action is that they interact with the transmembrane protein Zn-dependent metallopeptidase YvjB (also known as RseP) which belongs to the site-2 protease (S2P) protein family, serving as the receptor for these bacteriocins and that their interaction leads to pore formation in the bacterial cell membrane [106][75][85]. RseP is also known to be involved in bacterial stress response in

several species and deletion of *rseP* cause sensitivity towards extracytoplasmic stress, such as variation in temperature conditions and pH-levels in the environment [5][67][109][4]. The antimicrobial peptides of the LsbB-like bacteriocin family are effective against numerous Gram-positive bacteria including pathogenic strains of staphylococci and enterococci [86].

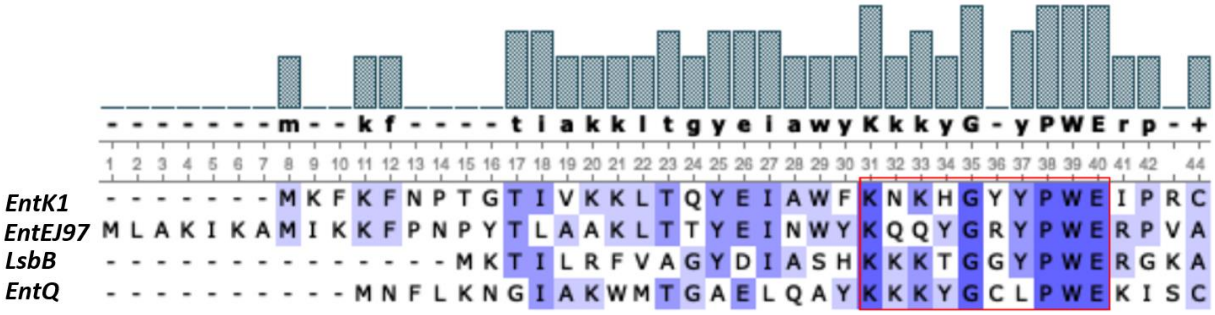

Figure 1.6. Multiple sequence alignment (MSA) of the four members from the LsbB-like bacteriocin family. Predicted by CLUSTALW alignment tool and exhibited using Unipro UGENE software v33.0. Consensus of identity is displayed above the sequences. Less conserved residues are colored light violet while highly conserved amino acids are highlighted with dark violet with KXXXGXXPWE motif at the C-terminal part (marked in red). N-terminal end vary in length and contain fewer conserved residues.

1.3.1. Enterocin K1

Enterocin K1 (EntK1) is a small linear and non-modified leaderless peptide which belongs to the LsbB-like bacteriocin family. EntK1 is produced by the bacterium *Enterococcus faecium* and has a relatively broad spectrum of activity against Gram-positive bacteria and especially effective against the strains of *E. faecium*. The peptide is composed of 37 amino acid residues with local folding within the peptide forming an α-helix structure from residue 8 – 24. The N-terminal part is amphiphilic and unsaturated in 1:1 tetrafluoroethylene (TFE) and water solution. However, EntK1 does not form secondary structure in water [85].

1.3.2. Enterocin EJ97

Enterocin EJ97 (EntEJ97) is a small linear and non-modified leaderless peptide which belongs to the LsbB-like bacteriocin family. EntEJ97 is a cationic bacteriocin produced by the bacterium *Enterococcus faecalis* and has a relatively broad spectrum of activity against Gram-positive bacteria, including *Listeria monocytogenes*. The peptide is composed of 44 amino acid residues and has a conserved C-terminal motif [50][85].

1.3.3. Hybrid bacteriocins Enterocin K1EJ97 and Enterocin EJ97K1

The newly bioengineered bacteriocins Enterocin K1EJ97 (EntK1EJ97) and Enterocin EJ97K1 (EntEJ97K1) are hybrids constructed by combining the N-terminal and C-terminal parts of the bacteriocins EntK1 and EntEJ97, designed at the Laboratory of Microbial Gene-technology (LMG). The hybrid bacteriocin EntK1EJ97 contains 37 amino acid residues and is composed of the N-terminal part of EntK1 and the C-terminal part of EntEJ97, while the opposite hybrid bacteriocin EntEJ97K1 contains 44 amino acid residues and is composed in the opposite fashion with the N-terminal part of EntEJ97 and the C-terminal part of EntK1. As seen in **Figure 1.7**, these two hybrid bacteriocins share a high degree of sequence identity and the common KXXXGXXPWE motif of the LsbB-like bacteriocin family.

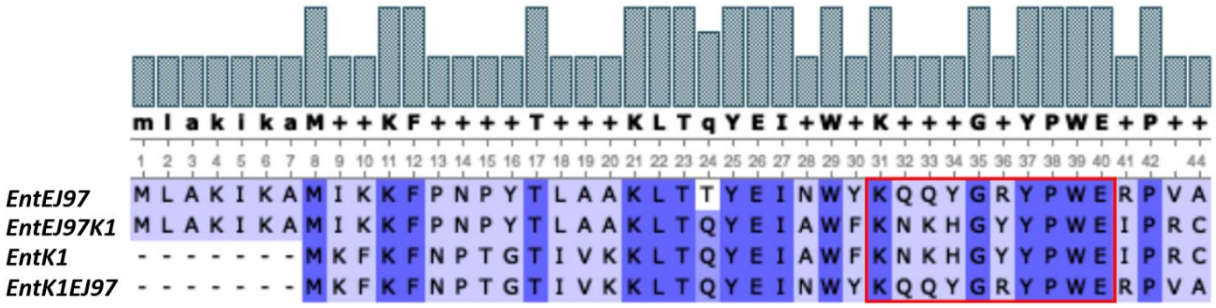

Figure 1.7. Multiple sequence alignment (MSA) of four bacteriocins used in this study. Predicted by CLUSTALW and showed in Unipro UGENE software v33.0. percentage identity is displayed above the sequences. Less conserved residues are colored light violet while highly preserved amino acids are highlighted with dark violet. Conserved KXXXGXXPWE motif is highlighted in red.

1.3.4. Nisin Z

Nisin Z belongs to the lantibiotic group of class I bacteriocins and is a well-known broad-spectrum polycyclic bacteriocin effective against many Gram-positive bacteria. Nisin Z is a bacteriocin produced by the bacterium *Lactococcus lactis*; it contains 34 amino acid residues and is often used as a food preservative during production to extend shelf lifetime of food products by suppressing spoilage by Gram-positive and pathogenic bacteria [98]. Its mechanism of action is that it creates pores in the bacterial cell membrane due to lipid II interactions [41].

1.3.5. Garvicin KS

Garvicin KS (GarKS) is a recently discovered leaderless multi-peptide (three peptide) bacteriocin produced by *Lactococcus garvieae* and belongs to the group of leaderless peptides of class II. GarKS contains the three unmodified peptides: GakA composed of 34 amino acid residues, GakB composed of 34 amino acid residues and GakC composed of 32 amino acid residues. These peptides have similar properties, displaying a slight antimicrobial effect alone. However, in combination, they are effective against a wide selection of bacterial pathogens in the genera *Listeria*, *Staphylococcus*, *Streptococcus*, *Bacillus* and *Enterococcus* [84]. Its mechanism of action is that it inhibits bacterial growth by pore formation in the bacterial cell membrane by a yet unknown mechanism.

1.3.6. Micrococcin P1

Micrococcin P1 (MP1) is a macrocyclic bacteriocin produced and post-translationally modified by *Staphylococcus equorum* and belongs to a group of heavily modified peptides known as thiopeptides. This antimicrobial peptide contains 14 amino acid residues with sulphur and nitrogen rich heterocyclic rings. MP1 is extremely hydrophobic and its mechanism of action is that it functions as an acceptor-site-specific inhibitor of ribosomal protein synthesis, effectively preventing the growth of pathogenic Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), but little or no effect against Gram-negative bacteria [37].

1.4. Staphylococci biofilms

Life within a biofilm probably represents the predominate mode of growth for microorganisms in most environments. Biofilms in general are the transition from free floating planktonic cells to sessile cells within biofilms and generally consist of cells that adhere to each other and often also to an abiotic or biotic surface and becomes embedded within an extracellular polymeric substance (EPS) which forms a gel-like matrix around the cell cluster. The extracellular matrix is generally composed of polysaccharides, proteins, lipids and nucleic acids (DNA and RNA) which provides structure and protection to the bacterial community within the biofilm [46][28][12][115][11][53][3].

In contrast to the planktonic cells, biofilms constitute a distinct growth phase [69]. Most microbes produce biofilms as a means of response to their unfavorable environmental conditions. Biofilms are produced as a result of coordinated gene expression of the individual cells via quorum sensing [2]. Quorum sensing regulation leads to an overall change in gene expression, increased virulence, and accelerating the gaining of antibiotic-resistance [102]. The sessile cells growing within a biofilm are physiologically distinct from the planktonic cells of the same species [80] and the microorganisms produce biofilms in response to several different factors such as cellular recognition of specific or non-specific attachment sites on a surface. In addition, nutritional factors and in some cases exposure of planktonic cells to subinhibitory concentrations of antimicrobial agents and environmental stress may also influence the transition from planktonic cells to sessile cells and subsequent biofilm production [61][55].

Biofilm-associated infections with staphylococci are usually not mixed with other bacterial species, this in contrast to many other medical biofilms such as multi-species dental plaque formation [8]. In addition, it is rare to find more than one strain in an infection. A possible explanation for this phenomenon is interspecies communication by quorum-sensing signals, which in staphylococci leads to interspecies inhibition of virulence factor expression [59]. Staphylococcal biofilms have a physiological status that is characterized by a general down-regulation of active cell processes, such as protein, DNA, and cell wall biosynthesis, which is typical of slow growing cells. Other metabolic changes can be interpreted as a switch to fermentative processes such as acetoin metabolism, resulting from the low oxygen

concentration in biofilms. Finally, the up-regulation of urease and the arginine deiminase pathway, which ultimately produce ammonia compounds, has been explained as a switch to limit the deleterious effects of the reduced pH associated with anaerobic growth conditions [11].

As seen in **Figure 1.8**, staphylococcal biofilm production and maturation has been proposed to develop through a two-step process involving an initial attachment and a subsequent maturation phase. These phases are physiologically distinct from each other and require phase-specific factors. The final detachment phase involves detachment and dispersal of single cells or cell clusters which may again colonize and produce biofilms at another location [83].

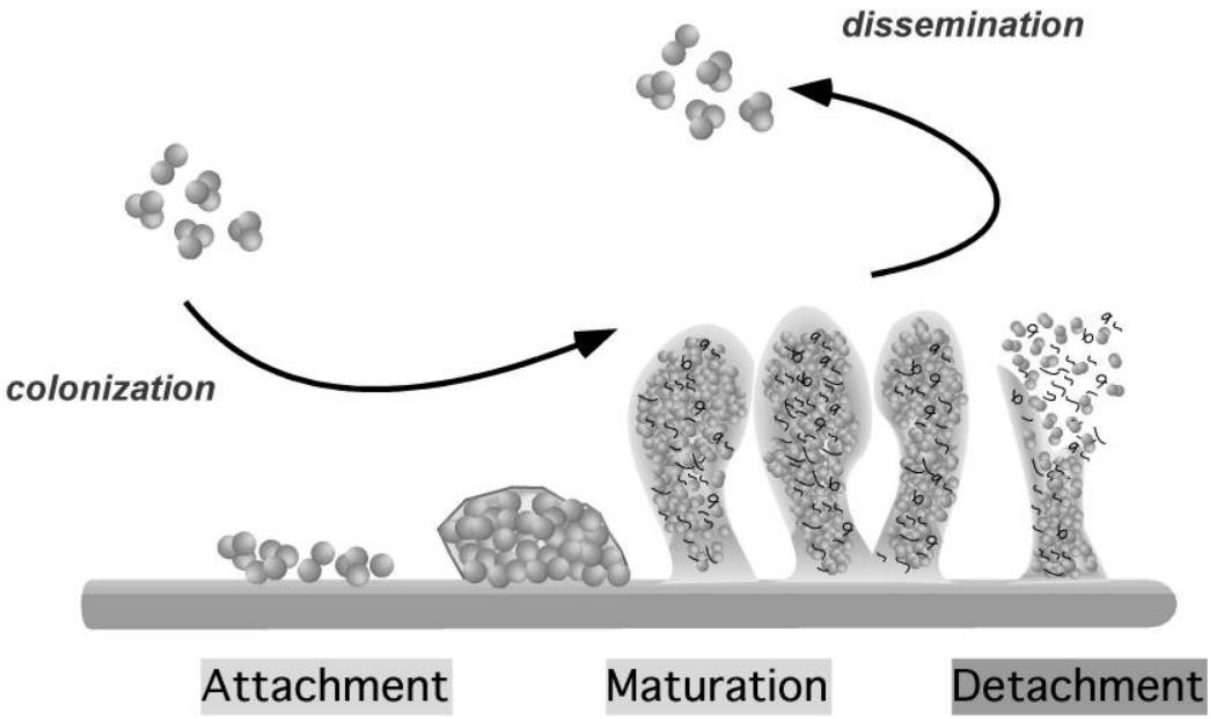

Figure 1.8. Proposed illustration for the different phases responsible for biofilm development in staphylococci. The figure shows the initial attachment of planktonic cells to a surface, which can occur on tissues or after covering of an abiotic surface by host matrix proteins in the human body (specific, protein-protein interaction), or directly to an abiotic surface (non-specific). The initial attachment phase is followed by biofilm growth, maturation and finally detachment. The figure is adapted from [83].

The sessile cells within the biofilm have the ability to share nutrients and genes by lateral or horizontal gene transfer. In addition, the cells are sheltered and protected from harmful factors in the environment such as desiccation, antimicrobial agents and the host's immune system [27][58]. Biofilm increase antibiotic resistance and often leads to persistent infections with a significant prolongation of the patient remission time [100]. The degree of observed resistance of the sessile cells within biofilms has been reported to be up to 10 to 1000-fold greater compared to planktonic cells of the same bacterial strain [21].

The ability to adhere to medical devices and subsequently produce biofilm is one of the major virulence factors associated with *S. haemolyticus* [36][35][20][48]. However, there is only limited knowledge about the initial attachment and the nature of *S. haemolyticus* biofilms. The initial attachment to a solid abiotic or biotic surface is thought to be facilitated by surface hydrophobicity [56][57], dedicated surface attachment proteins [32][40][54][66][103][110] and teichoic acid structure [52]. Several surface proteins have been identified which is associated with *Staphylococcus* ability to attach themselves to inert surfaces.

The initial step of *Staphylococcus* biofilm production within the human body is mediated through the attachment to human matrix proteins. Dozens of so-called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are expressed by *S. epidermidis* and *S. aureus.* These MSCRAMMs have affinity towards human matrix proteins such as fibrinogen or fibronectin and often combine binding capacity for several different matrix proteins [88]. These MSCRAMMs also share a common structure that includes an exposed binding domain, a cell wall spanning domain, which often has a repeat structure, and a domain that facilitates the covalent or non-covalent attachment to the bacterial surface [73]. The covalent attachment is catalyzed by a family of enzymes called sortases which links a conserved motif of the MSCRAMMs to peptidoglycan [71].

1.5. *Staphylococcus haemolyticus*

The bacterial species *S. haemoyticus* is a member of the genus *Staphylococcus* which consists of Gram-positive bacteria in the family staphylococcaceae in the order bacillales. Species within this genus are common pathogens of humans and other animals and are historically divided into two groups based on their ability to clot blood plasma (the coagulase reaction). The coagulase-positive staphylococci include *S. intermedius*, *S. hyicus* and *S. aureus* which is the most pathogenic of all known *Staphylococcus* species. The coagulase-negative staphylococci (CoNS) are now known to comprise over 30 species. The CoNS are generally common commensals of the bacterial skin flora, although some species can cause infections [47].

S. haemolyticus is a member of the CoNS [87] and is the second most clinically isolated of the CoNS from hospital-acquired human blood cultures, after *S. epidermidis* [35][101]. They are very much considered as an opportunistic pathogen which is part of the normal bacterial skin flora of humans, primates and domestic animals [36][44]. Infections caused by *S. haemolyticus* can be both local or systemic often associated with the insertion and implantation of medical devices during surgical procedures [43][91][111]. Human infections often include native valve endocarditis, sepsis, peritonitis and urinary tract, wound, bone, and joint infections [36][44][35][45]. Infrequent soft-tissue infections usually occur in immunocompromised patients [94].

S. haemolyticus has the highest level of antibiotic-resistance among the CoNS and multidrug resistance (MDR) against several different antibiotics is a very common property among them [48][49][23]. Various strains have been shown to possess resistance against one or more of these common classes of antibiotics: enicillins, cephalosporins, macrolides, quinolones, tetracyclines, aminoglycosides, and fosfomycin. Although resistance against vancomycin and teicoplanin is uncommon, glycopeptide-resistant strains has been reported [35][101][49][92]. The highly antibiotic-resistant phenotype among them and their ability to produce biofilms makes infections caused by *S. haemolyticus* quite difficult to treat [19].

1.6. The goal of this study

In this study the aim was to evaluate if the newly designed and bioengineered hybrid bacteriocin EntK1EJ97 could be applied as an effective alternative antimicrobial treatment to conventional antibiotics for the treatment of infections and biofilms caused by *S. haemolyticus*. As part of a collaboration with researchers from both India and Norway, we obtained six clinically isolated *S. haemolyticus* strains from India and seven clinically isolated *S. haemolyticus* strains from Norway. New knowledge was gained about the antimicrobial activity of EntK1EJ97 against *S. haemolyticus* and several other Gram-positive bacterial species from numerous genera. Throughout this study, it was observed that all the *S. haemolyticus* strains generated EntK1EJ97-resistant mutants which complicated the further bacteriocin treatment. For the purpose of preventing the generation of EntK1EJ97-resistant mutants and decrease the bacteriocin concentration needed to inhibit growth of *S. haemolyticus*, it was decided to apply combinatorial bacteriocin treatments with bacteriocins eliciting different mechanisms of action. Since *S. haemolyticus* is known to produce biofilms, it was important to determine the *in vitro* biofilm producing abilities of the strains and the degree of bacteriocin resistance going from free planktonic cells to sessile cells within biofilms. Through the work of Karolina Teresa Bartkiewicz (Master student, LMG), the transmembrane protein RseP was confirmed as the receptor for EntK1EJ97 (unpublished data). We hypothesized that the EntK1EJ97-resistant phenotype of the *S. haemolyticus* mutants was due to some mutations in the *rseP* gene encoding the RseP receptor. Because of the RseP receptors involvement in bacterial stress responses, it was interesting to investigate the temperature-stress responses of the EntK1EJ97-resistant mutants and perform sequencing of the *rseP* gene in order to determine if any mutations had occurred. In addition, morphological differences of the cell wall and cell membrane between the EntK1EJ97-sensitive wildtypes and the EntK1EJ97-resistant mutants was evaluated. We hypothesized that these investigations might perhaps explain the resistant phenotype of the *S. haemolyticus* mutants.

2. Materials and methods

2.1. Cultivation of bacterial strains and the bacteriocins used

All the bacterial strains used during this study was identified and verified by 16S rRNA sequencing. Cultivation of all the bacterial strains was done in liquid Brain Heart Infusion (BHI) medium (Oxoid) and incubated at 37°C without shaking in an incubation cabinet. All work with live bacteria was performed in a sterile flow hood. The supplied strains had been streaked out on BHI agar plates, and single colonies from these plates were picked with a sterile toothpick and used to inoculate 5 ml of liquid overnight (o/n) cultures. From each of these o/n cultures, a frozen stock was made by mixing 500 µl of 45% sterile glycerol (Sigma-Aldrich) with 1000 µl of o/n culture in cryotubes. The frozen stocks were stored at -80°C and served as the base of all subsequent cultivation of the strains which were performed in the following way:

A sterile toothpick was dipped in the still frozen stock and dropped into a new tube with 5 ml BHI. The tube was vortexed and incubated o/n at 37°C without shaking. These fresh o/n bacterial cultures were then used for the different experimental purposes during this study.

All the bacteriocins used during this study are listed in **Table 2.1** together with some general information about them and **Table 2.2** with their amino acid sequences. The bacteriocins EntK1, EntEJ97, EntK1EJ97, EntEJ97K1, nisin Z and GarKS was produced by Pepmic Co., LTD, China with > 95% purity. These bacteriocins were solubilized to the desired concentrations in 0.1% (vol/vol) trifluoroacetic acid (TFA). MP1 was purified to 95% purity at LMG from *B. altitudens* cell-free cultural liquid (1 L), applied to a Resource reverse-phase chromatography (RPC) column (1 ml) (GE Healthcare Biosciences) and connected to an AKTA purifier system (Amersham Pharmacia Biotech). A linear gradient of isopropanol (Merck) with 0.1% (vol/vol) TFA (buffer B) at a flow rate of 1.0 ml/min was used for elution. The identity of MP1 was confirmed by mass spectroscopy (MS) analysis with Ultraflex MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany). The concentration of MP1 was confirmed by HPLC (Dionex Ultimate 3000 LC system) using a commercial sample of known concentration (Cayman Chemical, Michigan USA) as a standard. All the bacteriocins were stored at -20°C until use.

Table 2.1. List of bacteriocins used in this study with their abbreviation (used in this thesis), spectrum of activity, a simplified mechanism of action associated with the bacteriocins and their stock concentration.

Bacteriocins	Abbreviation	Spectrum of activity	Mechanism of action	Stock concentration
Enterocin K1	EntK1	Narrow-spectrum	Pore formation due to RseP interactions	10.0 mg/ml
Enterocin EJ97	EntEJ97	Narrow-spectrum	Pore formation due to RseP interactions	10.0 mg/ml
Enterocin K1EJ97	EntK1EJ97	Unknown	Pore formation due to RseP interactions	10.0 mg/ml
Enterocin EJ97K1	EntEJ97K1	Unknown	Unknown	10.0 mg/ml
Nisin Z	---	Broad-spectrum	Pore formation due to lipid II interactions	1.0 mg/ml
Garvicin KS	GarKS	Broad-spectrum	Unknown	10.0 mg/ml
Micrococcin P1	MP1	Broad-spectrum	Inhibit bacterial translation due ribosome interactions	1.0 mg/ml

Table 2.2. List of the bacteriocins with their number of amino acid residues and amino acid sequences. The colored sequences indicate which part of the bacteriocins EntK1 and EntEJ97 and in which manner the hybrid bacteriocin EntK1EJ97 and the opposite hybrid bacteriocin is constructed. The sequences of nisin Z and MP1 are shown as their amino acid sequences before multiple post-translational modifications.

Bacteriocins		Amino acid residues	Amino acid sequence	Reference
EntK1		37	MKFKFNPTGTIVKKLTQYEIAWFKNKHGYYPWEIPRC	(Ovchinnikov et al., 2017)
EntEJ97		44	MLAKIKAMIKKFPNPYTLAAKLTTYEINWYKQQYGRYPWERPVA	(Galvez et al., 1998
EntK1EJ97		37	MKFKFNPTGTIVKKLTQYEINWYKQQYGRYPWERPVA	-
EntEJ97K1		44	MLAKIKAMIKKFPNPYTLAAKLTQYEIAWFKNKHGYYPWEIPRC	-
Nisin Z		34	ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK	(Shin et al., 2016)
GarKS:	**GakA**	34	MGAIIKAGAKIVGKGVLGGGASWLGWNVGEKIWK	(Ovchinnikov et al., 2016)
	GakB	34	MGAIIKAGAKIIGKGLLGGAAGGATYGGLKKIFG	
	GakC	32	MGAIIKAGAKIVGKGALTGGGVWLAEKLFGGK	
MP1		14	SCTTCVCTCSCCTT	(Degiacom et al., 2016)

2.2. Spectrum of activity against 51 strains-different genera and phylogenetic tree construction

The 51 strains-different genera listed in **Table 2.3** were cultivated and prepared by inoculating the strains in 5 ml of sterile BHI and allowed to grow o/n at 37°C without shaking. For each strain, a 50 µl aliquot of o/n culture (~1x10^8 CFU) was transferred in test tubes containing 5 ml of preheated (50 – 55°C) BHI soft agar. Tubes were vortexed for 3 seconds before they were poured on freshly made BHI agar plates. After the soft agar solidified, 3 µl of the bacteriocins EntK1, EntEJ97, EntEJ97K1, EntK1EJ97 and nisin Z were spotted with an equal distance between each other on the plates, as illustrated in **Figure 2.1**. Three concentrations of the bacteriocins were tested: 0.04 mg/ml, 0.2 mg/ml, and 1.0 mg/ml. The plates were incubated at 30°C for 24 h and the diameter of the inhibition zones where measured the next day. This work was performed in collaboration with Karolina Teresa Bartkiewicz (Master student, LMG). This experiment was reproduced in three independent experiments and the results are based on average values.

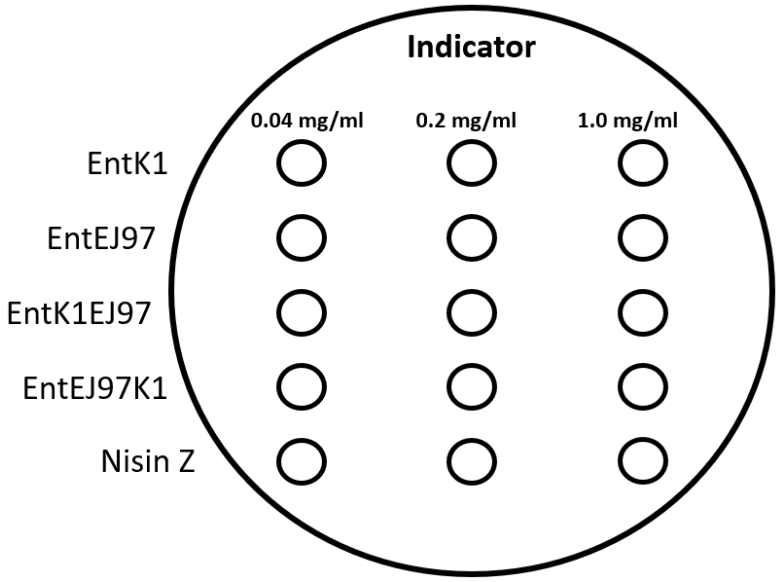

Figure 2.1. Schematic illustration of bacteriocin placement on the BHI agar plates against the indicators at concentrations of 0.04 mg/ml, 0.2 mg/ml and 1.0 mg/ml.

The average diameter of the inhibition zones where calculated and a scoring system was also implemented to show the degrees of bacterial inhibition by the different bacteriocins.

Scoring system:

0 = No inhibition

1 = Un-clear zone

2 = Clear small zone (<1.0 cm)

3 = Clear big zone (>1.0 cm)

"*" = Indicates zones with resistant mutant colonies

A Neighbor-joining phylogenetic tree was constructed by uploading the 16S rRNA sequences of the different bacterial species collected from the National Center for Biotechnology Information (NCBI) database and was uploaded to the software Molecular Evolutionary Genetics Analysis Version 10.1.6 (MEGA-X). Alignment was done by Muscle algorithm and Neighbor-joining constructed the best fitting tree based on the similarities within the 16S rRNA sequences of all the different bacterial species.

Table 2.3. List of bacterial indicator strains used during the spectrum of activity assay.

Species	Strain
Bacillus cereus	**LMGT2805**
Bacillus cereus	**LMGT2711**
Bacillus cereus	**LMGT2731**
Staphylococcus haemolyticus	**LMGT4068**
Staphylococcus simulans	**LMGT3233**
Staphylococcus arlettae	**LMGT4134**
Staphylococcus homonis	**LMGT3129**
Staphylococcus epidermidis	**LMGT3522**
Staphylococcus aureus	**LMGT3023**
Staphylococcus aureus	**LMGT3263**
Staphylococcus aureus	**LMGT3325**
Staphylococcus aureus	**LMGT3326**
Staphylococcus aureus	**LMGT3328**

Staphylococcus aureus	**LMGT3329**
Carnobacterium divergens	**LMGT2738**
Carnobacterium pisciola	**LMGT2332**
Enterococcus avium	**LMGT3465**
Enterococcus faecalis	**LMGT2333**
Enterococcus faecalis	**LMGT3088**
Enterococcus faecalis	**LMGT3330**
Enterococcus faecalis	**LMGT3331**
Enterococcus faecalis	**LMGT3332**
Enterococcus faecium	**LMGT2763**
Enterococcus faecium	**LMGT2772**
Enterococcus faecium	**LMGT2783**
Enterococcus faecium	**LMGT2876**
Lactobacillus curvatus	**LMGT2353**
Lactobacillus curvatus	**LMGT2355**
Lactobacillus plantarum	**LMGT2352**
Lactobacillus plantarum	**LMGT3125**
Lactobacillus sakei	**LMGT2361**
Lactobacillus sakei	**LMGT2380**
Lactobacillus salivarius	**LMGT2787**
Lactococcus garvieae	**LMGT3390**
Lactococcus lactis	**IL1403**
Lactococcus lactis	**LMGT2081**
Leuconostoc gelidum	**LMGT2386**
Listeria innocua	**LMGT2710**
Listeria innocua	**LMGT2785**
Listeria ivanovii	**LMGT2813**
Listeria monocytogenes	**LMGT2604**
Listeria monocytogenes	**LMGT2650**
Listeria monocytogenes	**LMGT2651**
Listeria monocytogenes	**LMGT2652**
Listeria monocytogenes	**LMGT2653**
Streptococcus dysgalactiae	**LMGT3890**
Streptococcus thermophilus	**LMGT3555**
Streptococcus uberis	**LMGT3912**
Streptococcus uberis	**LMGT3918**
Escherichia coli	**LMGT3590**
Escherichia coli	**LMGT3591**

2.3. Antimicrobial susceptibility test and generation of EntK1EJ97-resistant *S. haemolyticus* mutants

O/n cultures of six Indian *S. haemolyticus* strains together with one *S. aureus* (MRSA) strain were prepared by the same procedure as for the strains in the spectrum of activity assay and poured out on freshly made BHI agar plates. After the soft agar solidified, Antibiotic discs with antibiotics listed in **Table 2.4**, were placed with an equal distance from each other in a circle on top of the soft agar using a disc dispenser, whereas 3 µl of the hybrid bacteriocin EntK1EJ97 (diluted to concentrations of 0.2 mg/ml, and 1.0 mg/ml) were spotted in the middle of the plates. The BHI agar plates were then incubated at 37°C for 24 h; the next day the appearance of inhibition zones was confirmed visually, and their diameter was measured. This experiment was reproduced in three independent experiments and the results are based on average values.

Table 2.4. List of antibiotics used in this study and often used in treating infections caused by various pathogenic bacteria including staphylococci with their abbreviation, spectrum of activity, a simplified mechanism of action associated with the antibiotics and their stock concentration.

Antimicrobial agent	Abbreviation	Spectrum of activity	Mechanism of action	Antimicrobial concentration
Teicoplanin	Tec	Narrow-spectrum	Inhibit bacterial cell wall synthesis	30.0 µg/ml
Ciprofloxacin	Cip	Broad-spectrum	Inhibit bacterial DNA replication	5.0 µg/ml
Erythromycin	E	Broad-spectrum	Inhibit bacterial protein synthesis	15.0 µg/ml
Rifampicin	Rd	Broad-spectrum	Inhibit bacterial RNA synthesis	5.0 µg/ml
Vancomycin	Va	Narrow-spectrum	Inhibit bacterial cell wall synthesis	5.0 µg/ml
Penicillin G	P	Broad-spectrum	Inhibit bacterial cell wall synthesis	10.0 µg/ml

EntK1EJ97-resistant *S. haemolyticus* mutant colonies that appeared within the EntK1EJ97 (1.0 mg/ml dilutiuon) inhibition zones were picked. As illustrated in **Figure 2.2**, three resistant mutant colonies from each of the six Indian *S. haemolyticus* strains were selected and streaked on fresh BHI agar plates for isolation of pure cultures before frozen cultures were made and the mutant colonies were stored at -80°C until further use.

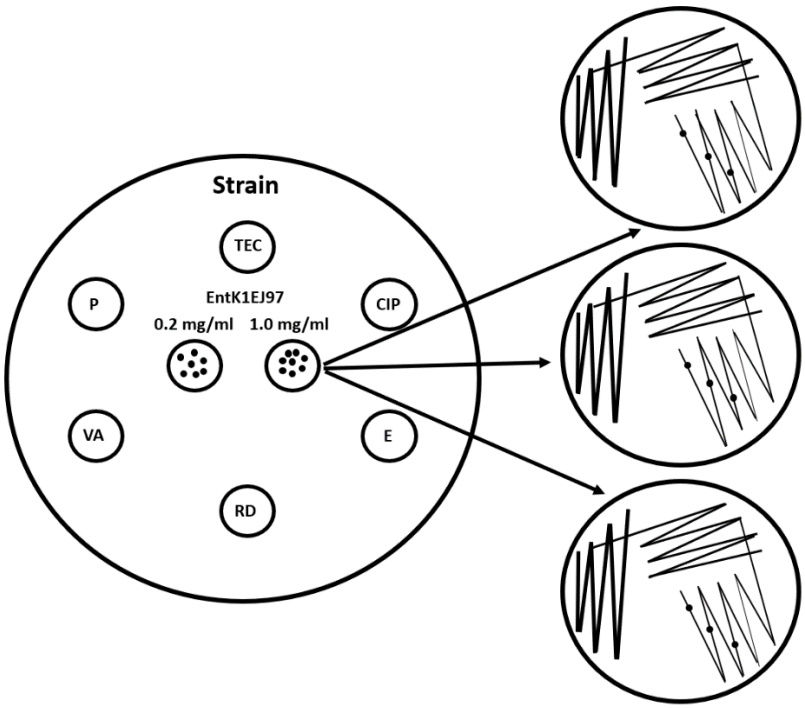

Figure 2.2. Schematic illustration of antimicrobial susceptibility test and method for isolating EntK1EJ97 resistant mutant colonies on BHI agar plates.

2.4. Mutant confirmation test

As illustrated in **Figure 2.3**, a 10 µl aliqiot of bacteriocin hybrid EntK1EJ97 (1.0 mg/ml) was streaked vertically in three lines across freshly made BHI agar plates and sterile toothpicks were used to transfer o/n wildtype *S. haemolyticus* strains and their respective mutants and streaked them horizontally across over the bacteriocin hybrid EntK1EJ97. The plates were then incubated at 37°C for 24 h to be observed the next day. This experiment was reproduced in three independent experiments and the results are based on average values.

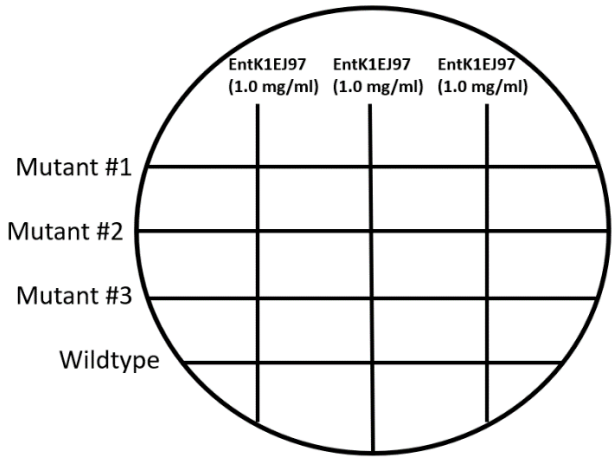

Figure 2.3. Schematic illustration of the mutant confirmation test showing vertical lines where the EntK1EJ97 at 1.0 mg/ml was streaked and horizontal lines where wildtypes with their respective mutants was streaked on BHI agar plates.

2.5. Microtiter plate biofilm assay

For the premise of determining *S. haemolyticus* biofilm producing abilities, a modified protocol of [81] was used. O/n cultures of six *S. haemolyticus* strains from India and six *S. haemolyticus* strains from Norway, together with one *S. aureus* (MRSA) strains as a positive control for biofilm formation and one *S. arlettae* strain as a negative control was tested. The strains were cultivated in BHI and were diluted 1:10 in 3% Tryptic Soy Broth (TSB) containing 1% glucose and 150 µl of the diluted strains were then transferred to the wells of a flat-bottom 96-well microtiter plate (one column for each strain) and the microtiter plates were incubated at the desired temperature at 37°C for 24 h with a transparent plastic cover tape over the wells of the microtiter plates to avoid evaporation of the medium. The next day, the planktonic cells in each of the wells were removed, by discarding the o/n medium and washing the biofilms with 160 µl saline solution (0.9% NaCl) at room temperature (RT) twice. The microtiter plates were then incubated at 55°C for 1 hour in order to fix the biofilms to the wells. After fixation, biofilms were stained by adding 150 µl 0.5% crystal violet solution in dH_2O to the wells and incubated for an additional 10 minutes at RT. Subsequently, the crystal violet solution was removed from the wells and the biofilms were washed twice with 0.9% NaCl. The biofilm-bound crystal violet was then eluted by adding 100 µl of absolute ethanol to the wells, and the plates were placed on a shaker for 10 minutes at 5 RPM. After the 10 minutes, the solution in the wells were transferred to a new microtiter plate and the

elution step was repeated once more. The amount of dye released by the biofilms, which is an indirect measure of the bacterial cell density, was measure spectrophotometrically at 600 nm using a SPECTROstar nano plate reader (BMG LABTECH) and pictures of the plates were also taken. This experiment was reproduced in three independent experiments and the results are based on average values. An average OD_{600} value <1.0 implies that the bacterial strain under investigation is a weak biofilm producer and an average OD value >1.0 implies that the bacterial strain is a strong biofilm producer.

2.6. Minimum inhibitory concentration (MIC) with single and combinatorial bacteriocin treatments against planktonic *S. haemolyticus* cells

Determination of the minimum inhibitory concentration (MIC_{50}) which refers to the concentration of antimicrobial agent causing 50% growth inhibition for the various single and combinatorial bacteriocin treatments against planktonic cells of the six Indian *S. haemolyticus* was performed by a modified version of the protocol [22]. The bacteriocins EntK1EJ97 and GarKS was diluted to a starting concentration of 100 µg/ml while MP1 was diluted to a starting concentration of 10 µg/ml. The various single and combinatorial bacteriocin treatments are listed in **Table 2.5** with the starting concentrations of each of the bacteriocins.

As seen in **Figure 2.4,** In flat-bottom 96-well microtiter plates, 135 µl sterile liquid BHI was transferred to the wells of the 2^{th} – 10^{th} column with a multichannel pipette, leaving the 1^{th} column medium-free. 150 µl BHI was transferred to the 12^{th} column and served as a negative control for planktonic cell growth (sterile medium) and 135 µl BHI with the appropriate amount of antimicrobial vehicle (TFA) was transferred to the wells of the 11^{th} column and served as a positive control for planktonic cell growth. 270 µl of the diluted bacteriocins was transferred to the appropriate wells of the 1^{th} column before starting serial diluting them 1:1 by gently pipetting up and down 10 times (without making bubbles) before transferring 135 µl of the diluted bacteriocins to the wells of the 2^{th} column and so on all the way to the wells of the 10^{th} column. Then, o/n bacterial cultures cultivated in BHI was diluted 1:5 in sterile BHI before transferring 15 µl of each bacterial strain to the appropriate

wells which already contained 135 µl diluted bacteriocin solution and TFA (positive control) and thus diluting the strains further 1:10 (so a total dilution of 1:50 for the strains).

The microtiter plates were then incubated at 37°C for the appropriate amount of time (5-, 24-, and 48 h) before measuring the OD_{600} in the SPECTROstar nano plate reader (BMG LABTECH) with shaking and determining the MIC_{50} values for each of the strains. This experiment was reproduced in three independent experiments and the results are based on average values.

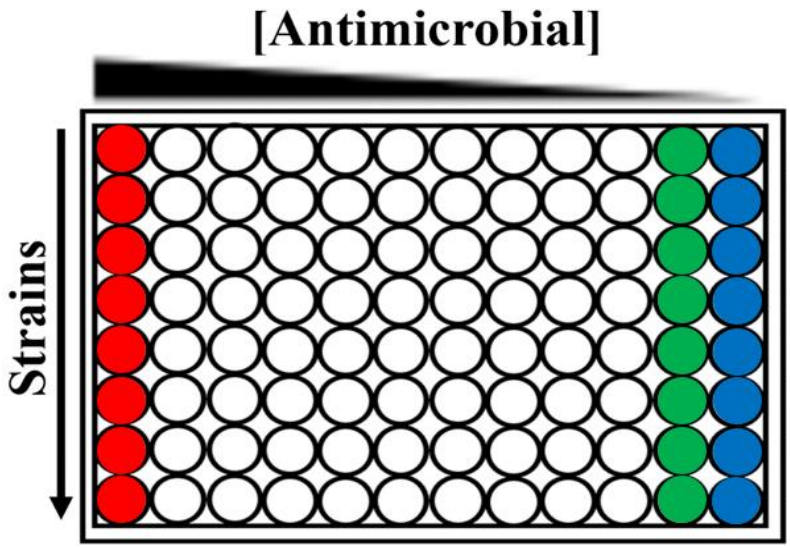

Figure 2.4. Minimum inhibitory concentration (MIC) assay in flat-bottom 96-well microtiter plate. Serial dilution of the single and combinatorial bacteriocin treatments started in the 1th column (red) and was serial diluted 1:1 until the 10th column. The 11th column (green) contained TFA and served as the positive control while the 12th column (blue) contained only BHI and served as a negative control.

Table 2.5. Single and combinatorial bacteriocin treatments against planktonic cells with 1:1 serial dilution starting concentrations.

Bacteriocin treatments	Start concentration (µg/ml)
EntK1EJ97	100
GarKS	100
MP1	10
EntK1EJ97	100
GarKS	100
GarKS	100
MP1	10
EntK1EJ97	100
MP1	10
EntK1EJ97	100
GarKS	100
MP1	10

2.7. Biofilm-oriented antimicrobial test (BOAT) with single and combinatorial bacteriocin treatments against sessile *S. haemolyticus* cells within biofilms

Performance of the biofilm-oriented antimicrobial test (BOAT) with single and combinatorial bacteriocin treatments was conducted by a modified version of the protocol [51]. The various bacterial strains were allowed to produce biofilms at 37°C for 24 h with the same procedure as for the biofilm assay. Preparation of the challenge plates was performed the following day and started with transferring 175 µl sterile liquid tryptic soy broth (TSB) medium in the appropriate numbers of wells in the microtiter plates, leaving the 1th row medium-free and a second set of wells was prepared for the controls. Then, in order to find a good starting concentration for the bacteriocin treatments, the highest average MIC_{100} value of EntK1EJ97 against the planktonic *S. haemolyticus* strains after 5 h incubation which refers to the concentration of antimicrobial agent causing 100% growth inhibition and the highest average MIC_{50} value of the combinatorial EntK1EJ97/GarKS/MP1 against the planktonic *S. haemolyticus* strains after 48 h incubation was multiplied 100-fold and mixed in falcon tubes. Preparation of a control mix with the same medium and the appropriate amount of antimicrobial vehicle 0.001% (vol/vol) TFA was performed in another falcon tube. Once the bacteriocins and control mix was ready, 350 µl of each mix was dispensed in the

appropriate number of wells in the 1th row of the challenge plates, as indicated in **Figure 2.5.** A 175 µl aliquot of the bacteriocins and control mix was then transferred from the 1th row to the 2th row and mixed by gently pipetting up and down 10 times and avoiding making bubbles. The bacteriocins and control mix was serially diluted 1:1 in this way until the 8th row of the challenge plates with a multichannel pipette.

The microtiter plates containing the 24 h biofilms were then removed from the incubator and the medium (100 µl) was aspired. The biofilms in all the wells was washed once with 100 µl of sterile saline solution (0.9% NaCl) at RT prior adding 150 µl of the bacteriocins and control mix to the appropriate wells of the plates. In this step, the bacteriocins and control mix was pipetted from the last row of the challenge plates (lowest concentration) and progressed up to the top using the same set of tips. Then, the plates containing the biofilm was placed back in the incubator at 37°C for the appropriate amount of time (5 h and 24 h for the single EntK1EJ97 bacteriocin treatment and 5-, 24-, and 48 h for the combinatorial EntK1EJ97/GarKS/MP1 bacteriocin treatment).

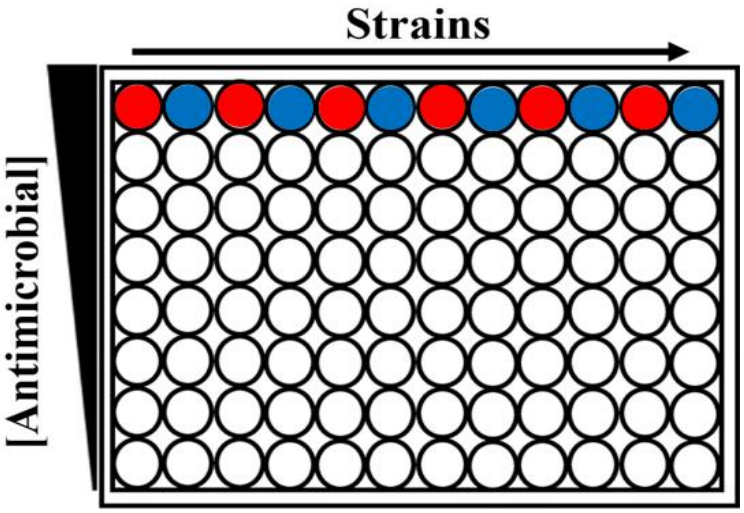

Figure 2.5. Biofilm-oriented antimicrobial test (BOAT) assay in flat-bottom 96-well microtiter plate. One column for the 1:1 serial diluted bacteriocin treatment (red) and one column for the control (blue) for each of the bacterial strains.

After the incubation with the single and combinatorial bacteriocin treatments, the residual metabolic activity of each strain was determined by using the metabolic indicator 2,3,5-triphenyltetrazolium chloride (TTC). In order to do this, the plates were removed from the incubator and the medium with the bacteriocins and control mix was removed, taking care to start from the first row of the plates (highest bacteriocin concentration, lowest theoretical viability) and moving towards the bottom. The biofilms were then washed twice with 150 µl sterile saline solution (0.9% NaCl) at RT. After the second wash, the residual saline buffer was completely removed prior adding 100 µl of TSB containing 0.025% (w/v) TTC before the plates were incubated at 37°C for an additional 5 h. The plates were then removed from the incubator and visually inspected for the development of red color (red formazan). The TTC:TSB solution was then removed and 200 µl of an ethanol:acetone mixture (70:30) was added to each well in order to extract the red formazan from the cells. The ethanol:acetone mixture was also added to the wells that developed no color. The plates were then wrapped in parafilm and left on the lab bench o/n at RT. The following day, OD_{492} was measured in the SPECTROstar nano plate reader (BMG LABTECH) and pictures of the plates was taken. The single bacteriocin treatment was replicated in three independent experiments and the results are based on average values. Due to the COVID-19 pandemic and the lab restrictions that followed, the combinatorial bacteriocin treatment was only performed once, although with a higher number of clinically isolated *S. haemolyticus* strains than the single bacteriocin treatment.

2.8. Temperature-stress assay with *S. haemolyticus* wildtypes vs. their respective EntK1EJ97-resistant mutants

The growth rate of *S. haemolyticus* wildtypes and their respective EntK1EJ97-resistant mutants was evaluated and performed in flat-bottom 96-well microtiter plates. O/n cultures of wildtype strains and their respective resistant mutants was diluted 1:100 in sterile BHI medium. A 200 µl aliquot for each of the strains (wildtypes and resistant mutants) was transferred to the wells of the microtiter plate and a transparent plastic cover tape was then placed over the wells to avoid evaporation of the medium. Next, the plates were placed in a Synergy H1 Hybrid reader (BioTek®) set to the desired temperature (37°C, 40°C, and 45°C) and the OD_{600} was measured every 30 minutes for 24 h with shaking. This experiment was reproduced in three independent experiments and the results are based on average values.

2.9. DNA isolation and *rseP* sequencing

The *rseP* was initially the point of interest as the RseP receptor has been established as the putative receptor for the bacteriocin EntK1EJ97 like the other bacteriocins of the LsbB-like bacteriocin family. DNA isolation from two o/n cultures of EntK1EJ97-sensitive *S. haemolyticus* strains as well as one of their respective resistant mutants was accomplished by combining FastPrep shaker with E.Z.N.A.® Plasmid Miniprep kit I (Omega Bio-Tek) according to the manufacturer's instruction. Cell lysis and DNA extraction was performed through alkaline SDS lysis assisted by mechanical bead-beating and by spin-columns specifically but reversibly binding DNA under optimized conditions. This allowed for contaminants and proteins to be washed away before eluting the extracted DNA. The *rseP* was amplified by PCR using species-specific *rseP* primers which was checked *in silico* using the primer sequence as a query in a nucleotide Basic Local Alignment Search Tool (BLAST) to ensure specificity towards *S. haemolyticus*. All the primers used in the PCR and sequencing was constructed by Karolina Teresa Bartkiewicz (Master student, LMG) during her work on *S. haemolyticus* and the primers was ordered from Invitrogen (Thermo Fisher Scientific).

The PCR reaction setup in this study followed the protocol for making the master mix, listed in **Table 2.6.** Primers was dissolved in DEPC treated Milli-Q, to a final concentration of 100 µM. From this stock, 20 µl was aliquoted and diluted 10-fold to a working solution of 10 µM for direct use in the PCR master mix.

Table 2.6. Reagents used to make the PCR master mix.

Components	25 µl RXN	Final Concentration
5X Q5 Reaction Buffer	5 µl	1X
10 mM dNTPs	0.5 µl	200 µM
10 µM Forward Primer	1.25 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	0.5 µM
Template DNA	2.0 µl	< 1.000 ng
Q5 High-Fidelity DNA Polymerase	0.25 µl	0.02 U/µl
Nuclease-Free Water	14.75 µl	

The PCR reaction was based on the program, shown in **Table 2.7** and was performed on a MyCycler™ thermal cycler (Bio-Rad). 2 µl of isolated gDNA was used as template. Following PCR, DNA quality and concentration were measured using NanoDrop 2000 (Thermo Scientific) before storing the samples at -20°C until further use. A minimal nucleic acid concentration of 50 ng/µl was desired, but the yield was usually well beyond this.

Table 2.7. PCR program used on a MyCycler™ thermal cycler (Bio-Rad).

Step	Temperature	Duration	Cycles
Initial denaturation	98 °C	1 min	
Denaturation	98 °C	30 s	30
Annealing	60 °C	30 s	
Elongation	72 °C	20 s/kb	
Final elongation	72 °C	2 min	
Hold	4 °C	∞	

The PCR results was analyzed on 1% Tris-acetate-EDTA (TAE) agarose gel using 2 µl PeqGREEN (Peqlab) DNA/RNA dye per 50 ml gel. Purple loading Dye (NEB) was used when loading samples on the gel and 1 kb Ladder (NEB) was used to estimate fragment size. The gel was run at 90 V for approximately 60 minutes.

PCR amplicons was purified using the NucleoSpin® Gel and PCR clean-up kit according to the manufacturer's instruction. Concentrations of the purified amplicons was measured by NanoDrop and diluted to within a range of 80 – 20 ng/µl in a nuclease free Eppendorf tube. Sequencing primers listed in **Table 2.8** was added to the purified amplicons for a final primer concentration of 2.5 nM, one primer per tube. The tubes were then sent to Eurofins Genomics for sequencing. The sequences were downloaded from the Eurofins Genomic website, and the corresponding chromatogram (in PDF format) was visually checked for any unambiguity in the sequence. The online bioinformatics tool Reverse Complement (Stothard 2000) (https://bioinformatics.org/sms/rev_comp.html) was used to generate a reverse complementary sequence from the reverse sequencing reaction. The online multiple sequence alignment tool ClustalW was then used to align the forward and reverse complement sequence. This consensus sequence was then used as a query in a nucleotide BLAST search.

Table 2.8. Primers used in PCR and sequencing of the *rseP* gene with their length as well as T_m.

Primer name	Sequence in 5' --> 3' direction	Length (nt)	Comments	T_m	Annotation
SH_LMGT4105_RseP_Seq_F1	5'-TTG AGT GCA CAT TTG ACT AGA C-3'	22	139 nt upstream	57°C	Forward primer for *S. haemolyticus rseP* amplification and sequencing
SH_LMGT4105_RseP_Seq_R1	5'-ACT CAA TGC TTC TGC TTC AGC-3'	21	84 nt downstream	59.6°C	Reverse primer for *S. haemolyticus rseP* amplification and sequencing
SH_LMGT4105_RseP_Seq_F2	5'-ATC GCT CCA CGA CAT CGA C-3'	19		62.4°C	Forward primer for *S. haemolyticus rseP* sequencing

SH_LMGT4105_RseP_Seq_R2	5’-GCT GCA GAC TGA ATG TCA TC-3’	20	58.6°C	Reverse primer for *S. haemolyticus rseP* sequencing
SH_LMGT4105_RseP_Seq_F3	5’-GAA CGA AAC TTT GTA TAC CAT CCG-3’	24	57.5°C	Forward primer for *S. haemolyticus rseP* seqeincing
SH_LMGT4105_RseP_Seq_R3	5’-ATG TAC TGG CAC TAA CAA ACT G-3’	22	57°C	Reverse primer for *S. haemolyticus rseP* sequencing
SH_LMGT4105_RseP_Seq_R4	5’-AAA TTC GAC CAC CAT CAA GTG C-3’	22	59.6°C	Reverse primer for *S. haemolyticus rseP* sequencing

LMGT4105 WT; ERS066291.7067_4_49.1

TTGAGTGCACATTTGACTAGACATAGTAGATGATGTAATTCTTAACTCCTTTTATTAAAAGTAAAATTCTTGAAT
AATTTAACCAAATCATAGATAATCTACATCGTTCTAATGAAAAAAGTAGAAATTTAATTTAATTGAGGTGTATCA
TGTGAGCTATTTAATCACTATTGTCTCATTTATGATCGTGTTTGGTGTACTTGTTACGGTACATGAATATGGTCA
CATGTTCTTTGCTAAGCGTGCTGGAATAATGTGTCCTGAATTCGCGATAGGTATGGGACCAAAAATATTTAGTT
TCCGTAAGAACGAAACTTTGTATACCATCCGTTTATTACCTGTAGGTGGTTATGTAAGAATGGCTGGAGACGGT
TTAGAAGAGCCACCAGTTGAACCAGGTATGAATGTTAAAGTAAAACTTAATGATAAAGATGAGATTACGCACA
TAATTTTAGATGATCAGCATAAATTTCAAAAGATAGAAGCAATTGAAGTTAAACAATGTGACTTCAAGGATGAT
TTGTACATTGAAGGTATTACTTCATATGATAATGAGCGACATCATTTTAATATTGCTGAAAAAGCATATTTTGTT
GAGAATGGTAGTTTAATTCAAATCGCTCCACGACATCGACAATTTGCACATAAGAAACCTTTACCCAAATTTTTA
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CACCTACTACCTCAGTGGGGCAATTAGCTGATCACTATCCAGCTCAACAAGCAGGATTAAAATCCGGAGATAA
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TAAGCAAACTAAATTAAATTCTGAGACGACGTACATTTTAGGTTTCCAACCAGAGAAAGAACATACTTTAATAA
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CGATATACAACGTTATTTCTTGTAAAATAAGGAGGATTAAGTGATGAAACAATCGAAAGTTTTCATACCAACTA
GGAGAGATGTCCCTGCTGAAGCAGAAGCATTGAGT

2.10. Transmission electron microscopy (TEM) images of *S. haemolyticus* wildtypes and their respective EntK1EJ97-resistant mutants

TEM is a microscopy technique in which a beam of electrons is transmitted through an ultrathin specimen and interacts with the structures of the specimen as it passes through it to form an image. TEM was applied in order to observe possible morphological differences between *S. haemolyticus* wildtypes and their respective EntK1EJ97-resistant mutants. The work was conducted in collaboration with Lene Cecilie Hermansen (Senior Engineer at IPV – imaging center, NMBU), Sofie S. Kristensen (PhD Candidate) and Karolina Teresa Bartkiewicz (Master student, LMG). The bacterial strains were picked for preparation in their stationary growth phase.

Preparation of planktonic *S. haemolyticus* wildtypes and their respective EntK1EJ97-resistant mutants and pictures taken with TEM was performed at the NMBU imaging center by the following procedure:

- **Fixation**

A 1.0 ml o/n bacterial suspension was transferred to Eppendorf tubes and centrifuged at 1.4 RPM for 10 minutes prior to discarding the supernatant. The cell pellet was washed with 1.0 ml PBS by pipetting up and down a few times and then centrifuged again at 1.4 RPM for 10 minutes. The supernatant was discarded, and the step was repeated once again. The cells were resuspended in 0.5 ml fixative solution and stored at 4°C until the next day.

- **Washing**

After o/n fixation, the fixative solution from the sample was collected in a waste bottle and the fixed cells was added 0.5 ml 0.04 M CaCo buffer which was discarded, and another 0.5 ml of the same buffer was added again.

- **Post fixation**

The 0.5 ml 0.04 M CaCo buffer was discarded from the cell sample and 2 drops of low melting agarose (warmed up until melting) was added in order to encapsulate the cells within agarose. After the agarose had solidified, the agarose containing the cell sample was transferred to a glass bottle and incubated in a mixed solution containing 1% OsO_4 and 0.1 M CaCo buffer for 70 minutes at RT. Then, the specimen was washed three times with 0.1 M CaCo buffer and incubated with shaking for 15 minutes each time.

- **Dehydration**

The buffer was discarded from the cell sample and 5.0 ml ethanol was added in different concentrations: 50%, 70%, 90%, 96% and 4 X 100%. The specimen was incubated for 15 minutes with shaking between discarding old and adding new ethanol solution.

- **Infiltration**

After discarding the last 100% ethanol solution from the specimen, 1.0 ml mixed solution containing LR White and 100% ethanol in ratio 1:3 was added and the specimen was incubated o/n at RT with shaking. The next day, the mixed solution was discarded and a new mixed solution in ratio 2:2 was added and incubated o/n at RT with shaking. The following day, the mixed solution was discarded and a new mixed solution in ratio 3:1 was added and incubated o/n at RT with shaking. Later the same day, the mixed solution was discarded, and 100% Resin was added and incubated o/n at RT with shaking.

- **Embedding**

The next day, the specimen was cleaved in two pieces and transferred into a capsule and completely covered with LR White. Then, the specimen was polymerized at 60°C for three days.

- **Sectioning and staining**

The process of ultramicrotomy was performed by cutting the specimen in slices of 60 nm thickness with a diamond knife. The selected slices were then collected onto a grid and stored in a grid cassette. The grid containing the sample was stained on a drop of mixed solution containing potassium permanganate and 40% uranyl acetate and incubated at RT for 10 minutes. Then, the grid was washed for 5 minutes on a drop of water which was repeated 10 times.

- **Imaging**

Imaging of the specimen was done in collaboration with Lene Cecilie Hermansen, Sofie S. Kristensen and Karolina Teresa Bartkiewicz. The specimen was observed under the transmission electron microscope with the use of the iTEM FEI software.

3. Results

3.1. The hybrid bacteriocin EntK1EJ97 is active against clinically important pathogenic bacteria in the genera *Staphylococcus* and *Enterococcus*

The bacteriocins spectrum of activity was determined against a sizable collection of 51 bacterial strains from 26 species of bacteria from various genera. The bacteriocin spectrum of activity as well as their potency against the indicators differed greatly both between genera and bacterial species within the same genera.

As seen in **Figure 3.1**, the antimicrobial activity of EntK1 and EntEJ97 was active against a broad spectrum of Gram-positive bacteria showing the second and fourth broadest spectrum of activity, respectively. The hybrid bacteriocin EntK1EJ97 showed the third broadest spectrum of activity with a slight change in its activity compared to EntK1 and EntEJ97. EntK1EJ97 was especially active against clinically important human pathogens within the *Enterococcus* genera and the *Staphylococcus* genera, particularly the *S. haemolyticus*. The opposite hybrid bacteriocin EntEJ97K1 showed the fifth broadest spectrum of activity and was mostly active against bacterial species within the *Lactobacillus* genera very similar to the activity of EntK1EJ97. Due to its broad spectrum of activity against Gram-positive bacteria, nisin Z served as a positive control and as expected was active against all but one bacterial species of the Gram-positive bacteria. Neither of the bacteriocins was active against the Gram-negative bacterium *Escherichia coli* which was also used to root the phylogenetic tree.

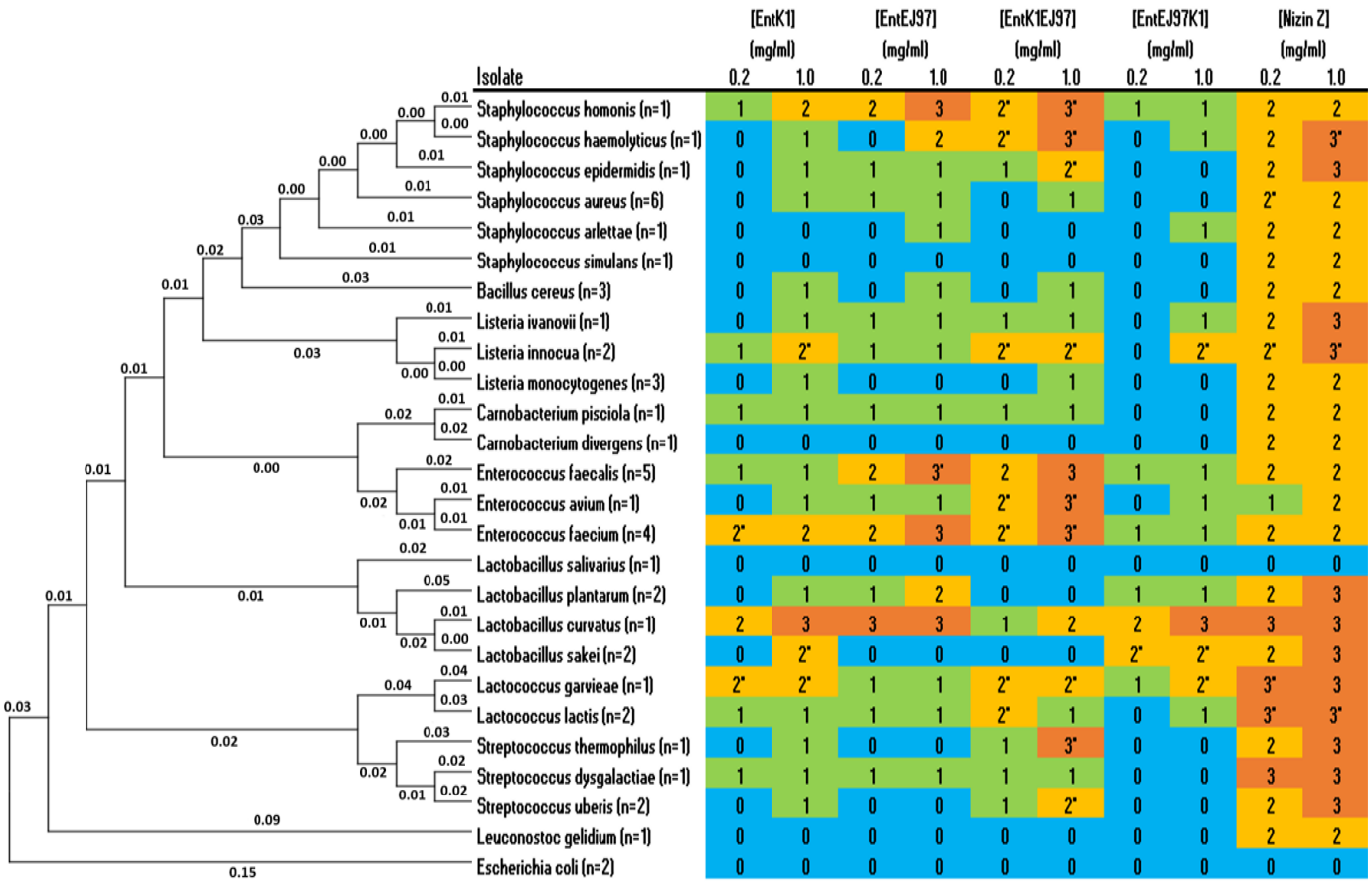

Figure 3.1. Graphical representation of spot-on-lawn spectrum of activity assay plotted onto the phylogenetic tree. This figure shows average scores for inhibitory activity of bacteriocin EntK1, EntEJ97, EntK1EJ97, EntEJ97K1 and nisin Z at concentrations of 0.2 mg/ml and 1.0 mg/ml correlated to evolutionary relationship among 26 different bacterial species (obtained in collaboration with Karolina Teresa Bartkiewicz). The result was observed after incubation at 30°C for 24 h. Nisin Z served as an inhibitory control due to its broad spectrum of activity. Scoring system: 0 = no inhibition (blue); 1 = unclear zone; 2 = small zone (<1 cm) (yellow); 3 = big zone (≥1 cm) (orange); "*" indicate zones containing resistant mutant colonies. Complete spectrum of activity results can be found in Appendix **A1**. The phylogenetic tree was generated by neighbor-joining algorithm.

As seen in **Table 2.2**, the hybrid bacteriocin EntK1EJ97 is composed of the N-terminal part of the bacteriocin EntK1 amino acid sequence and the C-terminal part of the bacteriocin EntEJ97 amino acid sequence while the opposite hybrid bacteriocin EntEJ97K1 is composed in the opposite fashion. Whether the N-terminal part of EntK1 is located on the hybrid bacteriocins N-terminus and the C-terminus part of the hybrid bacteriocins or vice versa seems to be crucial for the hybrid bacteriocins activity and affinity against the RseP receptor in which it interacts with.

3.2. EntK1EJ97 was most active against *S. haemolyticus* compared to various members of the LsbB-like bacteriocin family

Comparative study of EntK1EJ97 against various members of the LsbB-like bacteriocin family which included EntK1, EntEJ97, EntK1EJ97 and EntEJ97K1 was determined by the means of a spot-on-lawn assay in order to assess differences in susceptibility towards these bacteriocins after incubation at 37°C for 24 h. As part of a collaboration between LMG and researchers at the Blue Peter Public Health and Research Center in Hyderabad, India, we obtained six clinically isolated *S. haemolyticus* strains isolated from leprosy-associated plantar skin ulcers. These included the strains LMGT4068, LMGT4069, LMGT4070, LMGT4071, LMGT4072 and LMGT4073.

As seen in **Figure 3.2** and **Table 3.1**, EntEJ97 and EntK1EJ97 was most active against the six *S. haemolyticus* strains with EntK1EJ97 showing antimicrobial activity at all concentrations whereas EntEJ97 was really only active at the highest concentration. EntK1 and EntEJ97K1 did not show any antimicrobial activity at any of the concentrations.

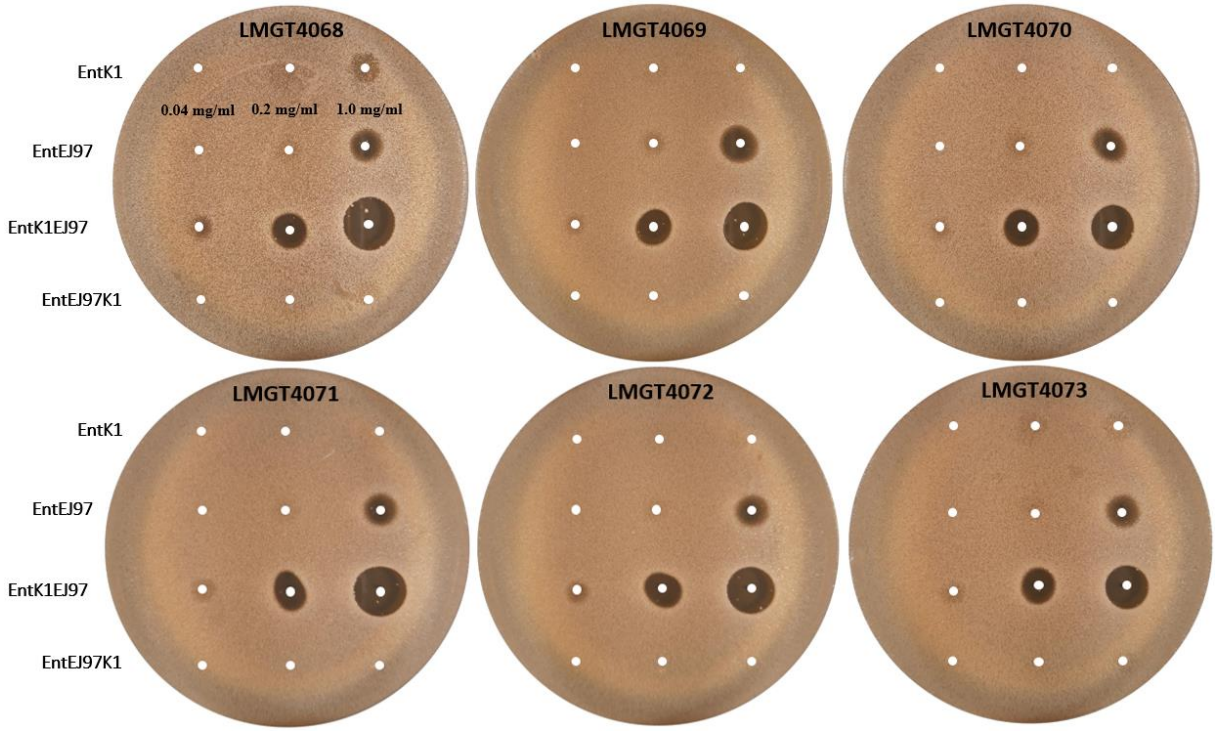

Figure 3.2 Representative spot-on-lawn results with 3 µl of the bacteriocins EntK1, EntEJ97, EntK1EJ97 and EntEJ97K1 at concentrations of 0.04 mg/ml, 0.2mg/ml and 1.0 mg/ml in the same orientation against the six Indian *S. haemolyticus* strains on BHI agar plates. The results were observed after incubation at 37°C for 24 h.

Table 3.1. The average score of inhibition zones produced by 3 µl spotted EntK1, EntEJ97, EntEJ97K1 and EntK1EJ97 at concentrations of 0.04 mg/ml, 0.2 mg/ml and 1.0 mg/ml against the six Indian *S. haemolyticus* strains. The results were observed after incubation at 37°C for 24 h. Scoring system: 0 = no inhibition (blue); 1 = unclear zone (green); 2 = small zone (<1 cm) (yellow); 3 = big zone (≥1 cm) (orange), "*" indicate zones containing resistant mutant colonies.

Bacteriocin	**[EntK1]** (mg/ml)			**[EntEJ97]** (mg/ml)			**[EntEJ97K1]** (mg/ml)			**[EntK1EJ97]** (mg/ml)		
Concentration	0.04	0.2	1.0	0.04	0.2	1.0	0.04	0.2	1.0	0.04	0.2	1.0
Strain												
LMGT4068	0	0	0	0	1	2*	0	0	0	1	2*	3*
LMGT4069	0	0	0	0	1	2*	0	0	0	1	2*	3*
LMGT4070	0	0	0	0	1	2*	0	0	0	1	2*	3*
LMGT4071	0	0	0	0	1	2*	0	0	0	1	2*	3*
LMGT4072	0	0	0	0	1	2*	0	0	0	1	2*	3*
LMGT4073	0	0	0	0	1	2*	0	0	0	1	2*	3*

Although EntEJ97 and EntK1EJ97 was quite active at inhibiting growth in all the strains, with EntK1EJ97 being the most potent bacteriocin, they both generated resistant mutant colonies within the inhibition zones. A particularly large number of resistant mutants was generated within the inhibition zones of EntK1EJ97. EntK1 and the opposite hybrid EntEJ97K1 did not display any activity against the tested *S. haemolyticus* strains. All these bacteriocins belongs to the LsbB-like bacteriocin family and their mechanism of action is believed to be the same in that they interact with the RseP receptor with various degree of affinity which leads to pore formation in the bacterial cell membrane [106][75][85].

3.3. EntK1EJ97 is a potential alternative to conventional antibiotics against *S. haemolyticus*

Comparative study of EntK1EJ97 as a possible alternative to conventional antibiotics against *S. haemolyticus* was determined by the means of an antimicrobial susceptibility test. A Kirby-Bauer antibiotic disc diffusion test was performed on the *S. haemolyticus* strains in order to compare the produced inhibition zones and antimicrobial activity of well-established antibiotics listed in **Table 3.2** often used in treating infections caused by various pathogenic bacteria including staphylococci with that of bacteriocin EntK1EJ97 after incubation at 37°C for 24 h. A methicillin-resistant *Staphylococcus aureus* (MRSA) strain (USA300) resistant

against beta-lactam antibiotics (penicillin derivatives such as methicillin and oxacillin) was also included as an antibiotic-resistant control strain.

Table 3.2. List of the various conventional antibiotics often used against pathogenic bacteria including staphylococci as well as bacteriocin EntK1EJ97 with some general information of their spectrum of activity, mechanism of action and concentrations.

Antimicrobial agent	Spectrum of activity	Mechanism of action	Concentration
Teicoplanin	Narrow-spectrum	Inhibit bacterial cell wall synthesis	30.0 µg/ml
Ciprofloxacin	Broad-spectrum	Inhibit bacterial DNA replication	5.0 µg/ml
Erythromycin	Broad-spectrum	Inhibit bacterial protein synthesis	15.0 µg/ml
Rifampicin	Broad-spectrum	Inhibit bacterial RNA synthesis	5.0 µg/ml
Vancomycin	Narrow-spectrum	Inhibit bacterial cell wall synthesis	5.0 µg/ml
Penicillin G	Broad-spectrum	Inhibit bacterial cell wall synthesis	10.0 µg/ml
EntK1EJ97	Narrow-spectrum	Inhibit proper integrity and function of bacterial cell membrane	0.2 mg/ml 1.0 mg/ml

As seen in **Figure 3.3** and **Table 3.3**, the antimicrobial susceptibility towards the various antimicrobial agents differed greatly. Among the tested antibiotics, all the strains resulted resistant to penicillin G and moderately susceptible to vancomycin and teicoplanin; whereas they showed a more heterogeneous pattern of susceptibility to the other antibiotic agents. As expected, the antibiotic-resistant *S. aureus* (MRSA) USA300 control strain showed only a weak susceptibility or complete resistance to the tested antibiotics. On the other hand, EntK1EJ97 was consistently showing the appearance of a clear inhibition zone for all the strains except *S. aureus* (MRSA) USA300 which was not susceptible to EntK1EJ97, although resistant mutants readily appeared which was also the case for some of the antibiotics. This is rationale basis for going into exploring the activity of EntK1EJ97 in combination with other bacteriocins.

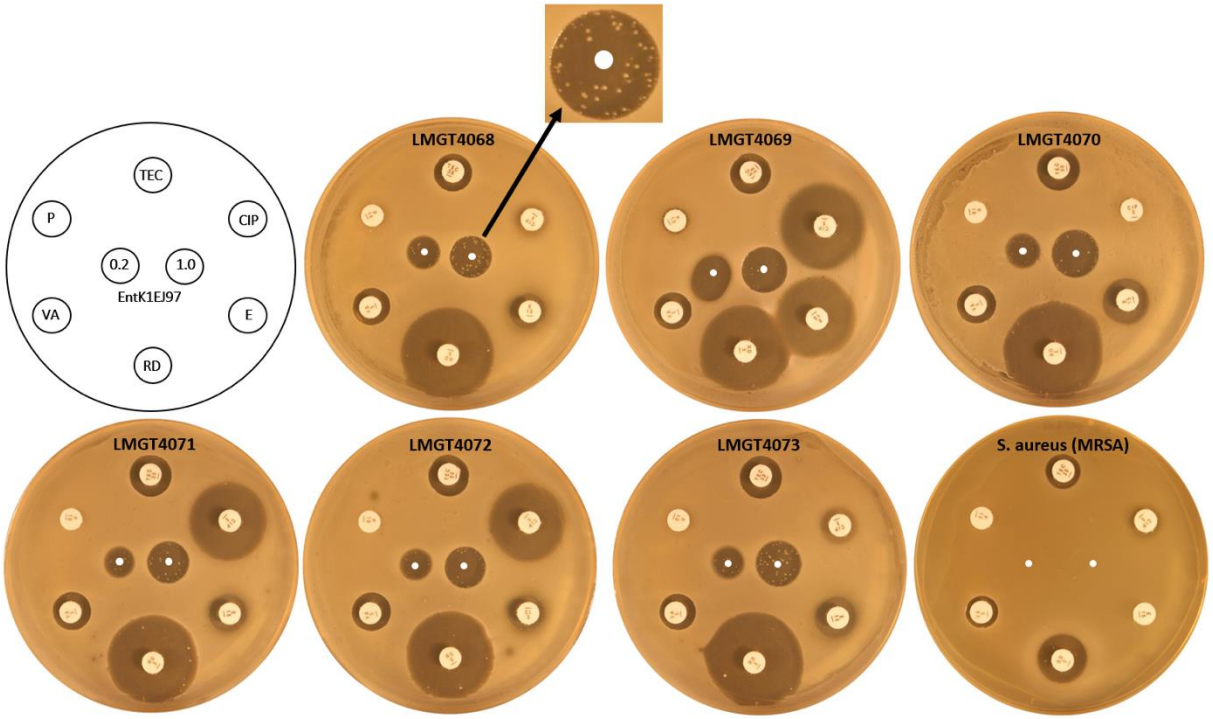

Figure 3.3. Comparative susceptibility of antimicrobial agents. Inhibition zones produced by discs of the antibiotics teicoplanin (TEC), ciprofloxacin (CIP), erythromycin (E), rifampicin (RD), vancomycin (VA), penicillin G (P) and 3 µl spotted EntK1EJ97 in the middle at concentrations of 0.2 mg/ml (left) and 1.0 mg/ml (right) against the six Indian *S. haemolyticus* strains together with *S. aureus* (MRSA) strain (USA300) on BHI agar plates. The results were observed after incubation at 37°C for 24 h.

Table 3.3. The average score of inhibition zones produced by discs of the antibiotics teicoplanin, ciprofloxacin, erythromycin, rifampicin, vancomycin, penicillin G and 3 µl spotted EntK1EJ97 at concentrations of 0.2 mg/ml and 1.0 mg/ml against the six Indian *S. haemolyticus* strains together with *S. aureus* (MRSA) strain (USA300). The results were observed after incubation at 37°C for 24 h. Scoring system: 0 = no inhibition (blue); 1 = unclear zone (green); 2 = small zone (<1 cm) (yellow); 3 = big zone (≥1 cm) (orange), "*" indicate inhibition zones containing resistant mutant colonies.

Antimicrobial	Teicoplanin	Ciprofloxacin	Erythromycin	Rifampicin	Vancomycin	Penicillin G	EntK1EJ97	
Concentration	30 µg/ml	5 µg/ml	15 µg/ml	5 µg/ml	5 µg/ml	10 µg/ml	0.2 mg/ml	1.0 mg/ml
Strain								
LMGT4068	3	3	3	3*	3	0	2*	3*
LMGT4069	3	3	3	3*	3	0	2*	3*
LMGT4070	3	0	3	3*	2	0	2*	3*
LMGT4071	3	3	3	3*	2	0	2*	3*
LMGT4072	3	3	3	3*	3	0	2*	3*
LMGT4073	3	2	3	3*	2	0	2*	3*
USA300	3	2	0	3*	2	0	0	0

Three of the EntK1EJ97-resistant mutant colonies within the 1.0 mg/ml inhibition zone from each of the six Indian *S. haemolyticus* strains was picked and re-streaked out on fresh BHI agar plates for the premise of isolating single EntK1EJ97-resistant mutant colonies for later use.

3.4. Resistance test confirms that the mutants from the *S. haemolyticus* strains are resistant against EntK1EJ97

The picked mutant colonies from the six Indian *S. haemolyticus* strains in previous experiment was subjected to a mutant confirmation test in which three lines was streaked with EntK1EJ97 at concentration of 1.0 mg/ml (vertical lines) and the six *S. haemolyticus* wildtypes together with their respective mutants was streaked (horizontal lines) across the lines containing EntK1EJ97. As seen in **Figure 3.4**, all the *S. haemolyticus* wildtypes was sensitive towards EntK1EJ97 indicated here by the separation in their lines across EntK1EJ97 and all their respective mutants was resistant indicated here by no separation in their lines across EntK1EJ97.

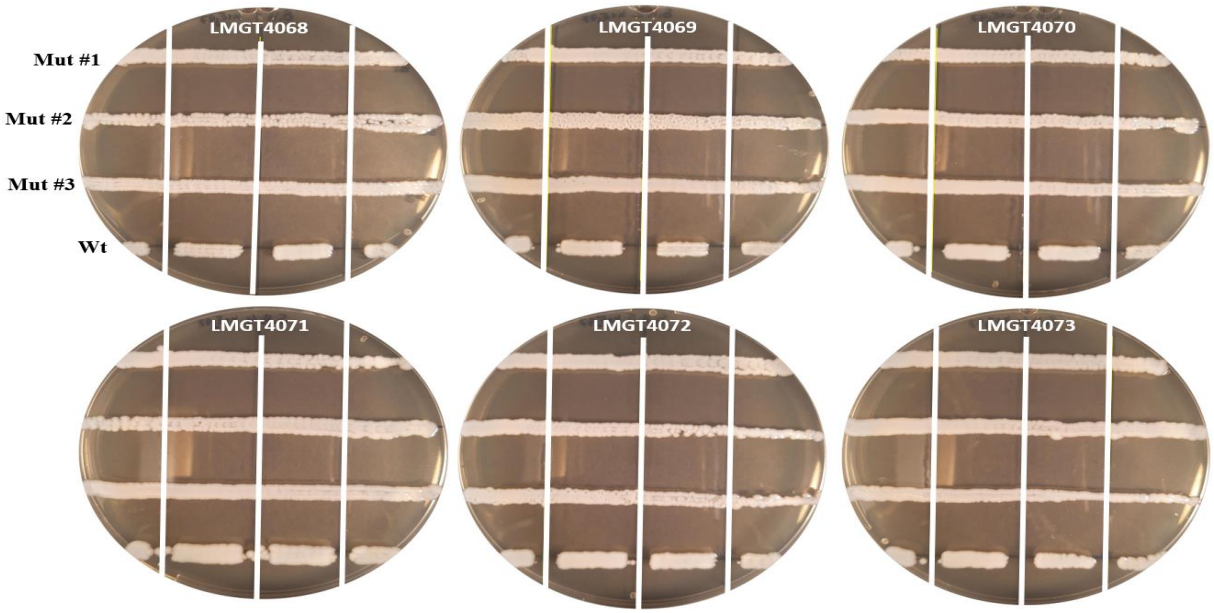

Figure 3.4. Visual representation of mutant confirmation test with EntK1EJ97 (1.0 mg/ml) streaked out vertically in three lines (white) against the six Indian *S. haemolyticus* wildtype (Wt) strains (bottom lines) with each of their respective EntK1EJ97 resistant mutants (Mut) (top three lines) on BHI agar plates. The results were observed after incubation at 37°C for 24 h.

3.5. Combinatorial bacteriocin treatment with GarKS and/or MP1 together with EntK1EJ97 might prevent generation of EntK1EJ97-resistant *S. haemolyticus* mutants

For the premise of minimizing the probability and prevent the generation of EntK1EJ97-resistant *S. haemolyticus* mutants, a spot-on-lawn assay was performed in which garvicin KS (GarKS) and micrococcin P1 (MP1) was chosen as good bacteriocin candidates especially considering that their mechanism of action differ from the bacteriocins of the LsbB-like family. EntK1, EntEJ97 and EntK1EJ97 was also included as a visual comparison and the evaluation was done after incubation at 37°C for 24 h. GarKS is a member of the leaderless bacteriocin family discovered at LMG and its mechanism of action is not yet fully understood, however what is known is that it like other leaderless bacteriocins causes pore formation in the bacterial cell membrane [84]. The mechanism for the thiopeptide MP1 is that it functions as a potent inhibitor of the bacterial ribosome and subsequently the translation of essential proteins for survival and reproduction [82][33].

As seen in **Figure 3.5** and **Table 3.4**, both GarKS and especially MP1 was quite effective against all the six Indian *S. haemolyticus* strains with MP1 showing antimicrobial activity at all concentrations whereas GarKS was really only effective at the highest concentration. However, there was no visual generation of either GarKS- or MP1-resistant mutant colonies within any of the inhibition zones. These results showed much promise for application of these two bacteriocins in various combinatorial bacteriocin treatments against *S. haemolyticus* and in preventing the generation of EntK1EJ97-resistant mutants.

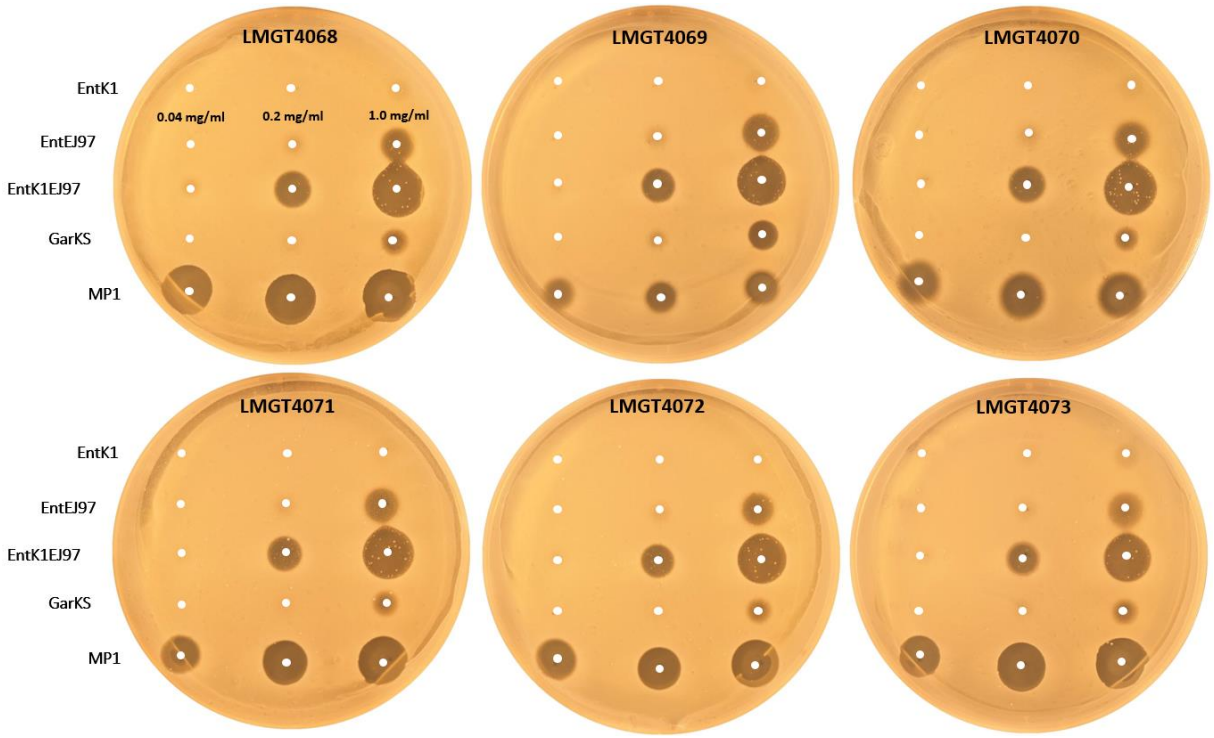

Figure 3.5. Representative spot-on-lawn results with 3 µl of the bacteriocins EntK1, EntEJ97, EntK1EJ97, GarKS and MP1 at concentrations of 0.04 mg/ml, 0.2mg/ml and 1.0 mg/ml in the same orientation against the six Indian *S. haemolyticus* strains on BHI agar plates. The results were observed after incubation at 37°C for 24 h.

Table 3.4. The average score of inhibition zones produced by 3 µl spotted EntK1, EntEJ97, EntK1EJ97, GarKS and MP1 at concentrations of 0.04 mg/ml, 0.2 mg/ml and 1.0 mg/ml against the six Indian *S. haemolyticus* strains. The results were observed after incubation at 37°C for 24 h. Scoring system: 0 = no inhibition (blue); 1 = unclear zone (green); 2 = small zone (<1 cm) (yellow); 3 = big zone (≥1 cm) (orange), "*" indicate zones containing resistant mutant colonies.

Bacteriocin	[EntK1] (mg/ml)			[EntEJ97] (mg/ml)			[EntK1EJ97] (mg/ml)			[GarKS] (mg/ml)			[MP1] (mg/ml)		
Concentration	0.04	0.2	1.0	0.04	0.2	1.0	0.04	0.2	1.0	0.04	0.2	1.0	0.04	0.2	1.0
Strain															
LMGT4068	0	0	0	0	1	2*	1	2*	3*	0	1	2	3	3	3
LMGT4069	0	0	0	0	1	2*	1	2*	3*	0	1	2	2	2	2
LMGT4070	0	0	0	0	1	2*	1	2*	3*	0	1	2	2	3	3
LMGT4071	0	0	0	0	1	2*	1	2*	3*	0	1	2	2	3	3
LMGT4072	0	0	0	0	1	2*	1	2*	3*	0	1	2	2	3	3
LMGT4073	0	0	0	0	1	2*	1	2*	3*	0	1	2	3	3	3

3.6. Planktonic *S. haemolyticus* cells was most effectively inhibited with triple combinatorial bacteriocin treatment without the generation of EntK1EJ97-resistant mutants

In an attempt to increase the antimicrobial potency, prevent the generation of EntK1EJ97-resistant mutants and lower the bacteriocin concentrations needed to inhibit growth of the planktonic cells of the six Indian *S. haemolyticus* strains, we explored the possibility to use the three bacteriocins in a combinatorial manner. In order to assess this, a 1:1 dilution series was applied with single and combinatorial treatments containing the bacteriocins EntK1EJ97, GarKS and MP1. The *S. haemolyticus* strains were exposed to the various single and combinatorial bacteriocin treatments at 37°C for 5-, 24-, and 48 h after which the *in vitro* minimum inhibitory concentration (MIC_{50}) was determined for each time period. Because the bacteriocins was used in different starting concentrations in the dilution series, an equivalent minimum inhibitory concentration ($EMIC_{50}$) value was implemented which directly correlates to the bacteriocin concentrations listed in **Table 3.5.** By analyzing the data using the ggplot package in Rstudio, box plots were generated to visualize any difference in the measured $EMIC_{50}$ among the strains.

Table 3.5. Equivalent concentration ([Eq]) in the 1:1 dilution series in which the values directly correlates to the concentration for each of the bacteriocins EntK1EJ97, GarKS and MP1 in µg/ml.

EntK1EJ97 [Eq]	[EntK1EJ97] (µg/ml)	GarKS [Eq]	[GarKS] (µg/ml)	MP1 [Eq]	[MP1] (µg/ml)
11	100.00	11	100.00	11	10.00
10	50.00	10	50.00	10	5.00
9	25.00	9	25.00	9	2.50
8	12.50	8	12.50	8	1.25
7	6.25	7	6.25	7	0.63
6	3.13	6	3.13	6	0.31
5	1.56	5	1.56	5	0.16
4	0.78	4	0.78	4	0.08
3	0.39	3	0.39	3	0.04
2	0.20	2	0.20	2	0.02
1	0.00	1	0.00	1	0.00

As seen in **Figure 3.6**, the various single and combinatorial bacteriocin treatments shows that the degree of inter-strain MIC_{50} variability displayed by the various treatments differed considerably. The MIC_{50} for all the strains after 5-, 24-, and 48 h incubation with the various single and combinatorial bacteriocin treatments are listed in Appendix **A2**, **A3** and **A4**, respectively. The strains displayed a decrease in susceptibility towards the treatments with increasing incubation time. EntK1EJ97 was only effective against all the strains after 5 h incubation with an average MIC_{50} of 0.78 µg/ml and was not effective after a prolonged incubation (24 h and 48 h) with an average MIC_{50} that exceeded the highest concentration of 100 µg/ml due to the generation and growth of the EntK1EJ97-resistant mutants. As for EntK1EJ97, neither of the GarKS and MP1 treatments was particularly effective after a prolonged incubation. Their effectiveness was significantly increased by applying them in various double combinations and displayed a strong synergistic effect towards the strains. However, the triple EntK1EJ97/GarKS/MP1 combination with all the bacteriocins was by far the most effective treatment after 5-, 24-, and 48 h with an average MIC_{50} ranging between ≤0.78/0.78/0.08 µg/ml after 5 h incubation and ≤6.25/6.25/0.63 µg/ml after 48 h incubation for the least sensitive strain LMGT4069. Not only did this treatment decrease the inter-strain MIC_{50} variability considerably, but it also reduced the concentration of each bacteriocin needed to inhibit bacterial growth and prevented the generation of EntK1EJ97-resistant *S. haemolyticus* mutants.

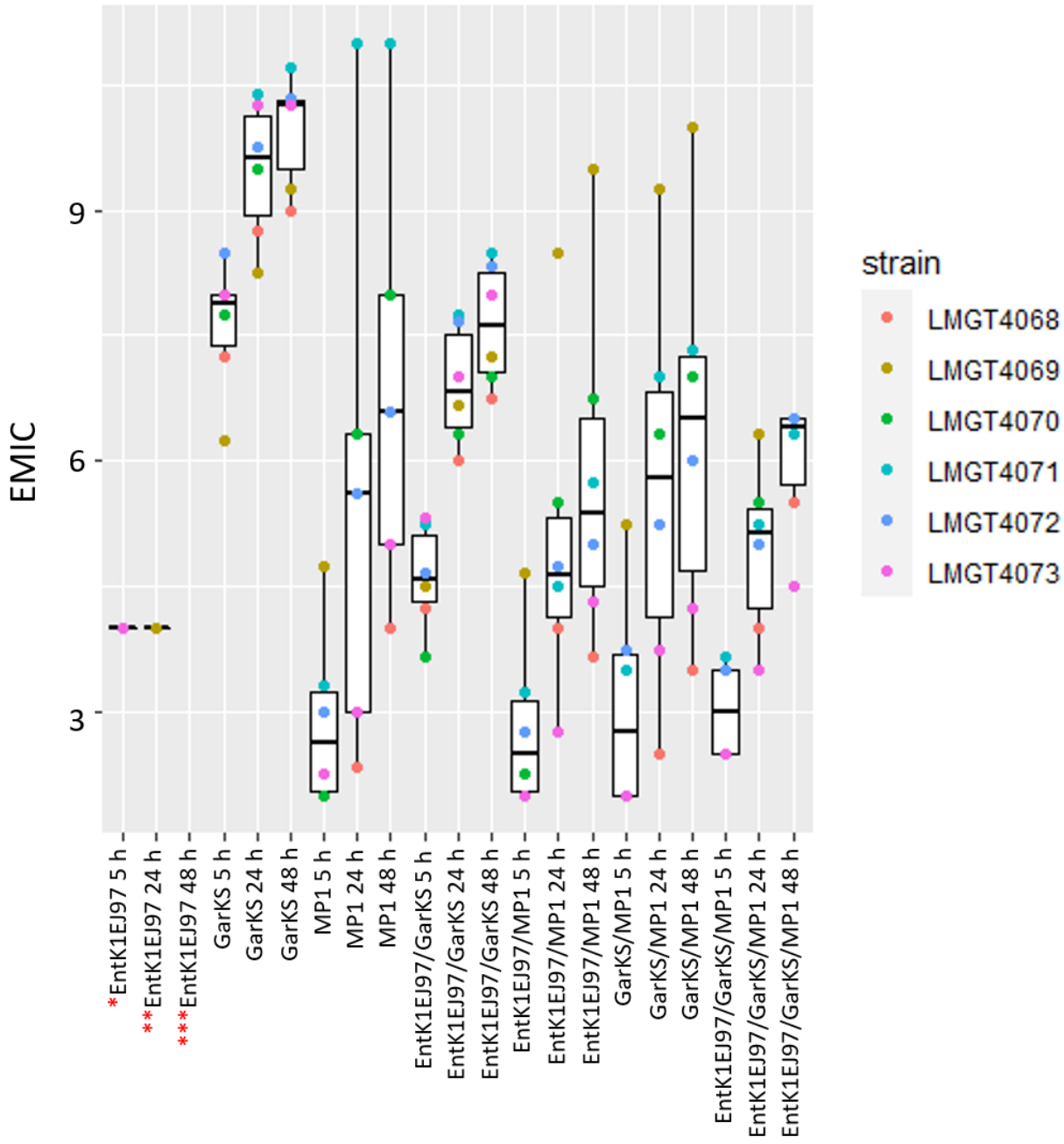

Figure 3.6. Box plot representation of average $EMIC_{50}$ values for planktonic cells of the six Indian *S. haemolyticus* strains after incubation at 37°C for 5-, 24-, and 48 h against a 1:1 dilution series with the bacteriocins EntK1EJ97, GarKS and MP1 in single and combinatorial bacteriocin treatments. "*" indicate that all the strains were sensitive at [EntK1EJ97] = 0.78 µg/ml ([Eq] = 4), "**" indicate that only the LMGT4069 strain showed inhibition at [EntK1EJ97] = 0.78 µg/ml and "***" indicate that all the strains were resistant.

3.7. *S. haemolyticus* is a strong *in vitro* biofilm producer

Having used the six *S. haemolyticus* clinical strains to determine the susceptibility of planktonic cells to the three bacteriocins and their combinations, we were now interested in analyzing the effects in an *in vitro* biofilm setting. To do this we extended the pool of *S. haemolyticus* strains included in the analysis to six more clinical strains obtained from the University Hospital in Tromsø, Norway (LMGT4106, LMGT4097, LMGT4103, LMGT4113, LMGT4105 and LMGT4115). Since the ability to produce biofilms is one of the main virulence factors associated with *S. haemolyticus* that makes infections caused by *S. haemolyticus* difficult to treat [35], it was important to evaluate the biofilm producing capacities of our *S. haemolyticus* strains. The *S. aureus* (MRSA) strain USA300 served as a control for a known strong biofilm producer [110] and the *S. arlettae* strain LMGT4059 served as a control for a known weak biofilm producer and was also included.

The *in vitro* determination of the strains ability to produce biofilms was performed by the means of a biofilm assay incubated at 37°C for 24 h with liquid sterile $TSB_{1\%GLU}$ medium which has been reported to enhance *S. haemolyticus* biofilm production [48]. The sessile cells within the biofilms was stained with 0.5% crystal violet which was extracted from the cell's peptidoglycan composed cell wall and measured at OD_{600} which corresponds to the amount of biofilm produced by each strain.

A representative result of the strains biofilm producing abilities in which the determination was based on can be seen in **Figure 3.7** were all the extracted crystal violet was measured. An OD_{600} threshold of >1.0 was set as a standard value and if the strains had an average OD_{600} >1.0 they were considered as strong biofilm producers, but if the strains had an average OD_{600} <1.0 they were considered as weak biofilm producers. All the strains mainly produced biofilms at the bottom and at the air-liquid interface within the wells of the microtiter plates as seen in **Figure 3.8.**

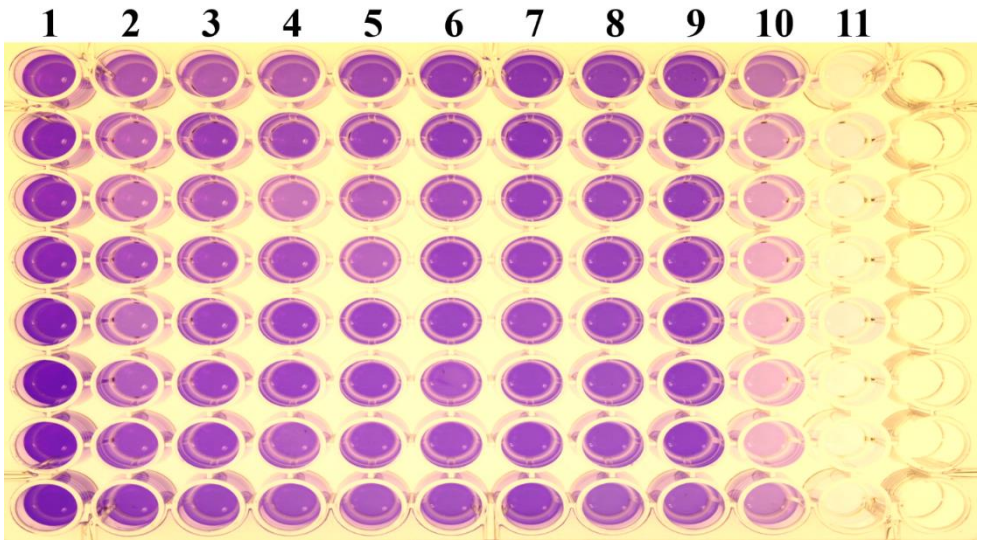

Figure 3.7. Representative result of crystal violet 0.5% extraction from biofilm assay at 37°C for 24 h with the six Indian *S. haemolyticus* strains (1 – 6), the Norwegian *S. haemolyticus* strains LMGT4113 and LMGT4105 (7 – 8), the *S. aureus* (MRSA) strain USA300 (9), the *S. arlettae* strain LMGT4059 (10) and negative control (11) in a flat-bottom 96-well microtiter plate viewed in a top-down perspective.

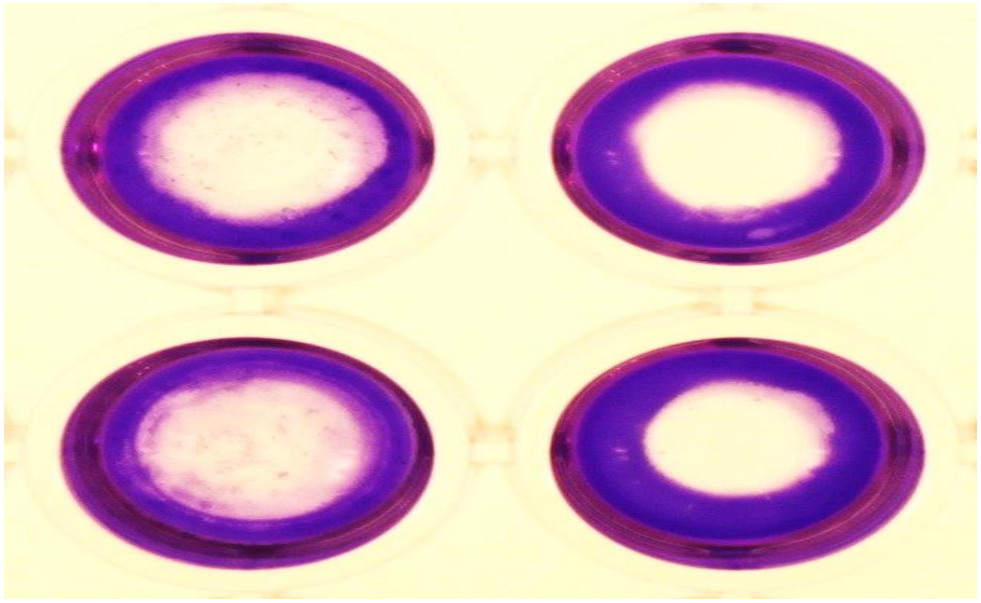

Figure 3.8. Representative result of *S. haemolyticus* biofilms stained with crystal violet 0.5% before extraction of the dye showing that the *S. haemolyticus* strains mainly produced biofilms at the bottom and at the air-liquid interface within the wells of the microtiter plates viewed in a top-down perspective.

As seen in **Figure 3.9**, all the *S. haemolyticus* strains from both India and Norway was determined to be very strong *in vitro* biofilm producers under optimal growth conditions with an overall average OD_{600} of 3.18. Almost all the strains except LMGT4106 was as strong as or slightly stronger biofilm producers than the *S. aureus* (MRSA) strain USA300 which was confirmed to be a very strong biofilm producer with an average OD_{600} of 3.27 and much stronger than the *S. arlettae* strain LMGT4059 which was confirmed to be a weak biofilm producer with an average OD_{600} of 0.49.

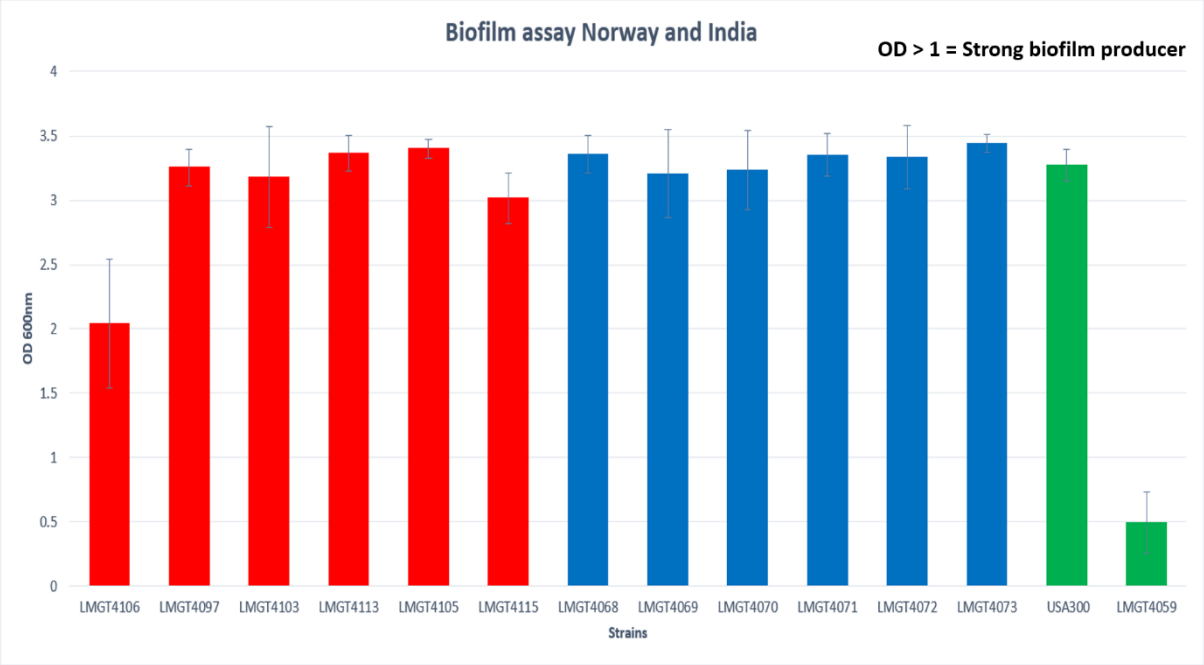

Figure 3.9. Biofilm assay performed in flat-bottom 96-well microtiter plates with $TSB_{1\%GLU}$ incubated at 37°C for 24 h showing average OD_{600} of extracted crystal violet 0.5% from the six Norwegian *S. haemolyticus* strains (red), the six Indian *S. haemolyticus* strains (blue), the *S. aureus* (MRSA) strain USA300 as a control for a strong biofilm producer (green) and the *S. arlettae* strain LMGT4059 as a control for a weak biofilm producer (green).

In addition to the previous biofilm assay, it was also interesting to determine whether the respective EntK1EJ97-resistant *S. haemolyticus* mutants isolated from the Indian strains were also capable of producing biofilms as their wildtypes. The biofilm assay was replicated and as seen in **Figure 3.10**, all the EntK1EJ97-resistant mutants was also very strong biofilm producers with the same ability to produce biofilms as their respective wildtypes with an overall average OD_{600} of 3.08.

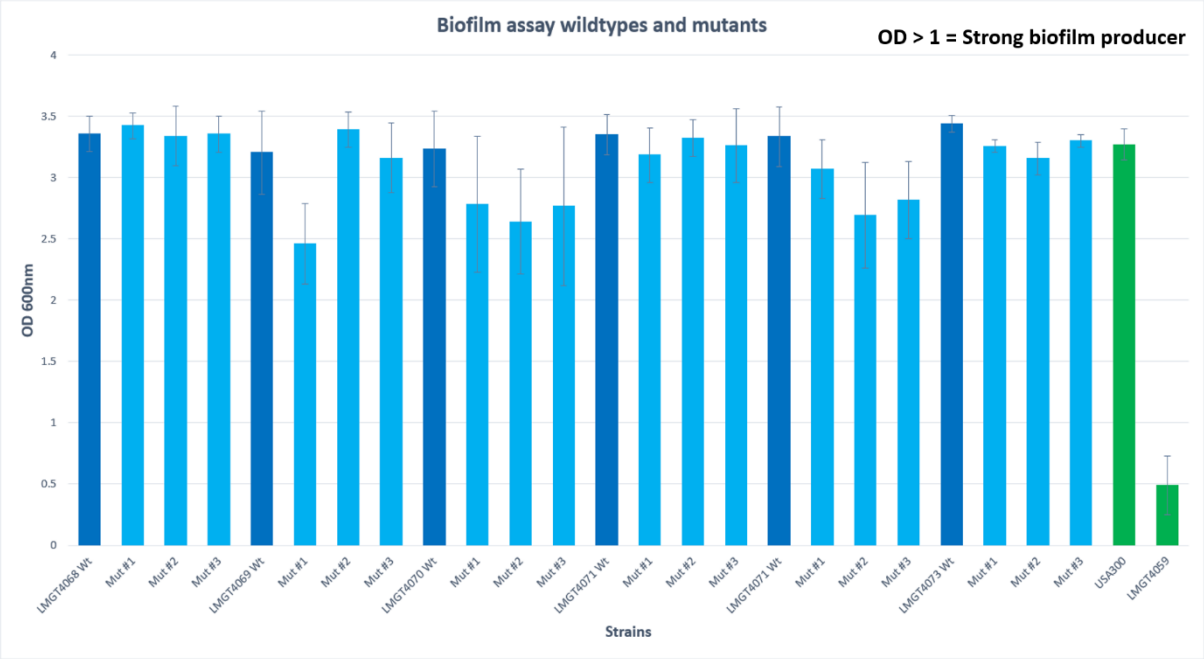

Figure 3.10. Biofilm assay performed in flat-bottom 96-well microtiter plates with $TSB_{1\%GLU}$ incubated at 37°C for 24 h showing average OD_{600} of extracted crystal violet 0.5% from the six Indian *S. haemolyticus* wildtype (Wt) strains (blue) with their respective EntK1EJ97-resistant mutants (Mut) (light blue), the *S. aureus* (MRSA) strain USA300 as a control for a strong biofilm producer (green) and the *S. arlettae* strain LMGT4059 as a control for a weak biofilm producer (green).

3.8. Growth of EntK1EJ97-resistant sessile *S. haemolyticus* mutants within biofilms after prolonged incubation with EntK1EJ97 treatment.

Determining the *in vitro* MIC_{50} for the single EntK1EJ97 bacteriocin treatment against sessile *S. haemolyticus* cells within biofilms was performed by the means of a biofilm-oriented antimicrobial test (BOAT) [51] with a 1:1 dilution series. The *S. haemolyticus* strains was exposed to the EntK1EJ97 treatment at 37°C for 5 h and 24 h after which the *in vitro* minimum inhibitory concentration (MIC_{50}) was determined for each strain after these time periods. In addition to the six Indian *S. haemolyticus* strains, the Norwegian *S. haemolyticus* strain LMGT4115, the *S. aureus* (MRSA) strain USA300 and the *S. arlettae* strain LMGT4059 were also included.

Because sessile cells within biofilms are known to display a higher degree of antimicrobial resistance than their planktonic cells [21], it was decided to multiply the average MIC_{100} of 1.56 µg/ml after 5 h EntK1EJ97 exposure of the planktonic cells in the previous MIC assay 100-fold in order to find a suitable bacteriocin starting concentration for the dilution series

listed in **Table 3.6.** For the premise of determining the strains MIC_{50} after 5 h and 24 h listed in Appendix **A5**, the residual metabolic activity for each strain was determined by using the metabolic indicator 2,3,5-triphenyltetrazolium chloride (TTC) which is metabolized by living cells and develops a red colored compound (red formazan) which again can be used as a measure of the strains metabolic activity and viability at OD_{492}.

Table 3.6. Dilution numbers in the 1:1 bacteriocin EntK1EJ97 dilution series with their corresponding concentrations (µg/ml).

Dilution nr.	[EntK1EJ97] (µg/ml)
D0	160.00
D1	80.00
D2	40.00
D3	20.00
D4	10.00
D5	5.00
D6	2.50
D7	1.25

As seen in **Figure 3.11**, EntK1EJ97 was only effective against the *S. haemolyticus* strains after the 5 h incubation with an inter-strain MIC_{50} variability ranging between ≥5.00 µg/ml for the most sensitive strain LMGT4068 and ≥80.00 µg/ml for the least sensitive strain LMGT4069. These results show that the sessile cells of the least sensitive strain LMGT4069 was approximately 102.4-fold more resistant than its planktonic cells after 5 h incubation with the EntK1EJ97 treatment. However, EntK1EJ97 was not effective against any of the *S. haemolyticus* strains after the prolonged 24 h incubation with an average MIC_{50} that exceeded the highest concentration. This was due to the generation and growth of the EntK1EJ97-resistant mutants at a prolonged incubation time period which is consistent with the results of the EntK1EJ97 treatment against the planktonic *S. haemolyticus* cells in which they were treated with EntK1EJ97 for 24 h and 48 h.

The results also show that the strains LMGT4069, LMGT4071 and LMGT4072 displayed a slightly enhanced ability to grow in the presence of both subinhibitory EntK1EJ97 concentrations of 2.50 µg/ml and 1.25 µg/ml after 5 h and the highest EntK1EJ97 concentrations of 160.00 µg/ml and 80.00 µg/ml after 24 h compared to the other strains and the control only treated with TFA.

The *S. aureus* (MRSA) strain USA300 and the *S. arlettae* strain LMGT4059 was not inhibited at any of the EntK1EJ97 treatment exposures with an average MIC_{50} that exceeded the highest concentration of 160.00 µg/ml and could not be determined. This might be explained by the lower susceptibility of these strains which was also demonstrated for the *S. aureus* (MRSA) strain in the comparative antimicrobial susceptibility test with the various conventional antibiotics and bacteriocin EntK1EJ97.

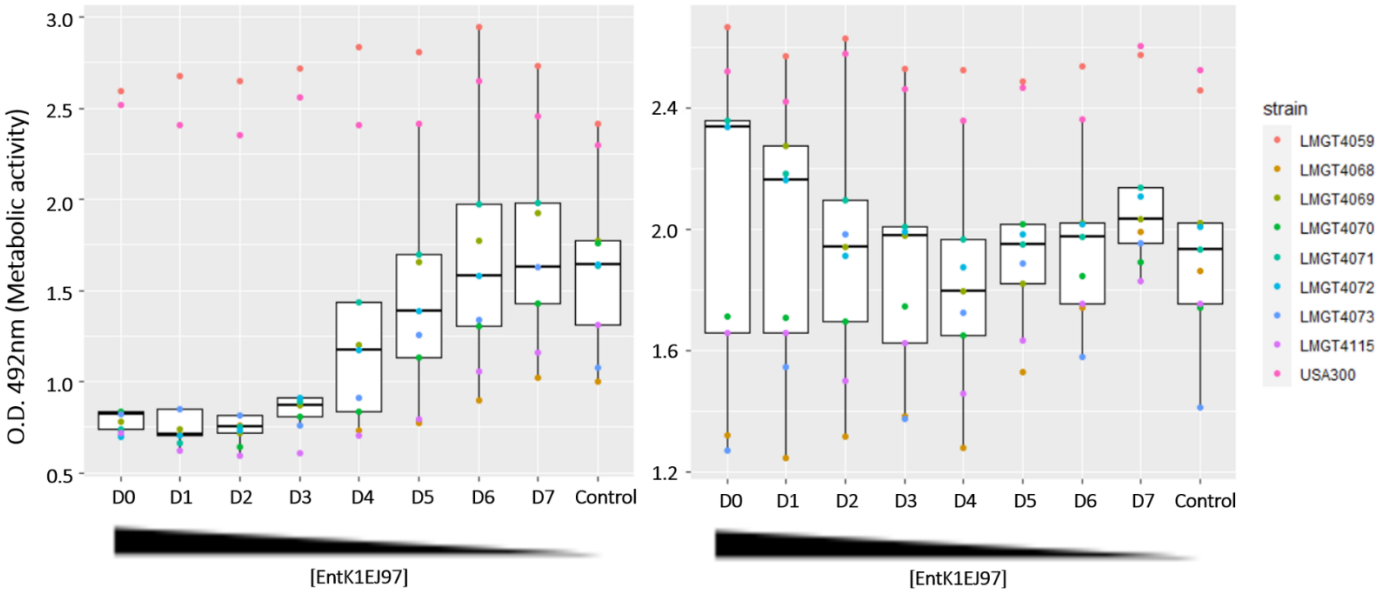

Figure 3.11. Box plot representation of average OD_{492} for the sessile cells of the six Indian *S. haemolyticus* strains, the Norwegian *S. haemolyticus* strain LMGT4115, the *S. aureus* (MRSA) strain USA300 and the *S. arlettae* strain LMGT4059 after 5 h (left) and 24 h (right) exposure to a 1:1 dilution series with bacteriocin EntK1EJ97.

3.9. Sessile *S. haemolyticus* cells within biofilms was effectively inhibited with triple combinatorial bacteriocin treatment without the generation of EntK1EJ97-resistant mutants

Determining the *in vitro* MIC_{50} for the triple combinatorial EntK1EJ97/GarKS/MP1 bacteriocin treatment against sessile *S. haemolyticus* cells within biofilms was performed by following the exact procedure as for the previous BOAT experiment. The *S. haemolyticus* strains was exposed to the EntK1EJ97/GarKS/MP1 treatment at 37°C for 5-, 24-, and 48 h after which the *in vitro* minimum inhibitory concentration (MIC_{50}) was determined for each strain after these time periods.

For the premise of treating the sessile cells, the EntK1EJ97/GarKS/MP1 bacteriocin treatment which was most effective against the planktonic cells of the *S. haemolyticus* strains was chosen. In addition to the six Indian strain, the seven Norwegian *S. haemolyticus* strains LMGT4106, LMGT4097, LMGT4103, LMGT4113, LMGT4105, LMGT4115 and LMGT4132 were also included in this experiment. In order to find a suitable starting concentration for the 1:1 dilution series listed in **Table 3.7**, the highest average MIC_{50} of ≤6.25/6.25/0.63 µg/ml for the same bacteriocin treatment against the planktonic cells after 48 h was multiplied 100-fold.

Table 3.7. Dilution numbers in the 1:1 triple combinatorial EntK1EJ97/GarKS/MP1 bacteriocin treatment dilution series with their corresponding concentrations (µg/ml).

Dilution nr.	[EntK1EJ97] (µg/ml)	Dilution nr.	[GarKS] (µg/ml)	Dilution nr.	[MP1] (µg/ml)
D0	625.00	D0	625.00	D0	62.50
D1	312.50	D1	312.50	D1	31.25
D2	156.25	D2	156.25	D2	15.63
D3	78.13	D3	78.13	D3	7.81
D4	39.06	D4	39.06	D4	3.91
D5	19.53	D5	19.53	D5	1.95
D6	9.77	D6	9.77	D6	0.98
D7	4.88	D7	4.88	D7	0.49

As seen in **Figure 3.12** and **Figure 3.13** the EntK1EJ97/GarKS/MP1 treatment displayed a strong synergistic effect against the sessile cells as for the planktonic cells although the degree of inter-strain MIC_{50} variability differed considerably. The strains displayed a decrease in susceptibility towards the treatment with increasing incubation time. EntK1EJ97/GarKS/MP1 was highly effective against the *S. haemolyticus* strains after 5-, 24-, and 48 h treatments with an average MIC_{50} ranging between ≥19.53/19.53/1.95 µg/ml after 5 h incubation and ≥78.13/78.13/7.81 µg/ml after 48 h incubation for the least sensitive strain LMGT4069. The MIC_{50} for all the strains after 5-, 24-, and 48 h incubation with the triple combinatorial EntK1EJ97/GarKS/MP1 bacteriocin treatment are listed in Appendix **A6.**

These results show that the sessile cells of the least sensitive strain LMGT4069 was approximately 12.5-fold more resistant than its planktonic cells after 48 h incubation with the EntK1EJ97/GarKS/MP1 treatment. In comparison to the single EntK1EJ97 treatment, EntK1EJ97GarKS/MP1 was approximately 4.1-fold more effective after 5 h incubation and reduced the concentration of each bacteriocin needed to inhibit bacterial growth considerably and prevented the generation of EntK1EJ97-resistant *S. haemolyticus* mutants.

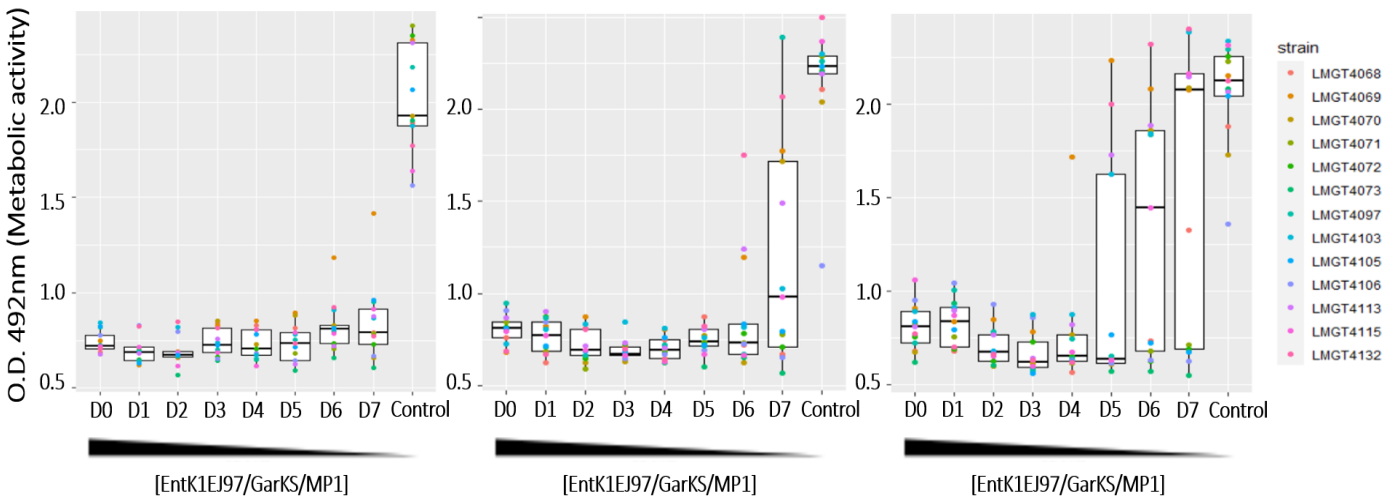

Figure 3.12. Box plot representation of average OD_{492} for the sessile cells of the six Indian *S. haemolyticus* strains and the seven Norwegian *S. haemolyticus* strains after incubation at 37°C for 5 h (left), 24 h (middle), and 48 h (right) exposure to a 1:1 dilution series with the triple combinatorial EntK1EJ97/GarKS/MP1 bacteriocin treatment.

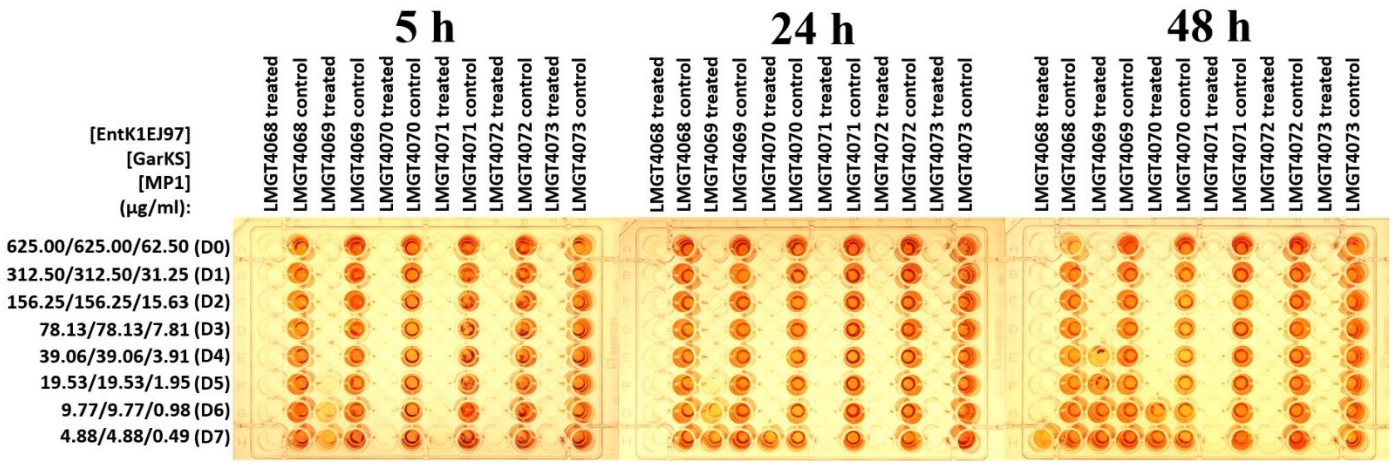

Figure 3.13. Representative result of the biofilm-oriented antimicrobial test (BOAT) with the triple combinatorial EntK1EJ97/GarKS/MP1 bacteriocin treatment and control with TFA in a 1:1 dilution series against the six Indian *S. haemolyticus* strains in which they were treated to for 5-, 24-, and 48 h. Red colored wells indicate the strains TTC metabolic activity and viability at decreasing bacteriocin concentrations.

3.10. *S. haemolyticus* wildtypes and their respective EntK1EJ97-resistant mutants adapt equally well under stressful temperature conditions

As a standard preliminary phenotypical test for mutant strains, since the RseP receptor has been found to be the receptor for bacteriocin EntK1EJ97 and also known to be involved in stress response in various bacteria [5]. A mutation in this Zn-dependent protease may putatively convert bacterial sensitivity to increasing temperature conditions. Therefore, it was interesting to investigate whether there were any significant differences in the growth rate of the EntK1EJ97-sensitive *S. haemolyticus* wildtypes and their respective EntK1EJ97-resistant mutants at increasing temperatures. For the premise of this temperature-stress assay, it was decided that the six Indian *S. haemolyticus* wildtype strains with their respective EntK1EJ97-resistant mutants were to be exposed to temperature conditions of 37°C, 40°C, and 45°C for a 24 h time period while the OD_{600} was measured every 30 minutes.

As seen in **Figure 3.14**, **Figure 3.15** and **Figure 3.16**, the result shows that there were no significant differences in the growth rate and the adaptive ability of the wildtypes and their respective mutants at either the lag-, exponential-, or stationary growth phase. These results were not expected had there been any significant mutation within the *rseP* gene which probably would had led to a decreased ability to deal with the stressful temperature conditions.

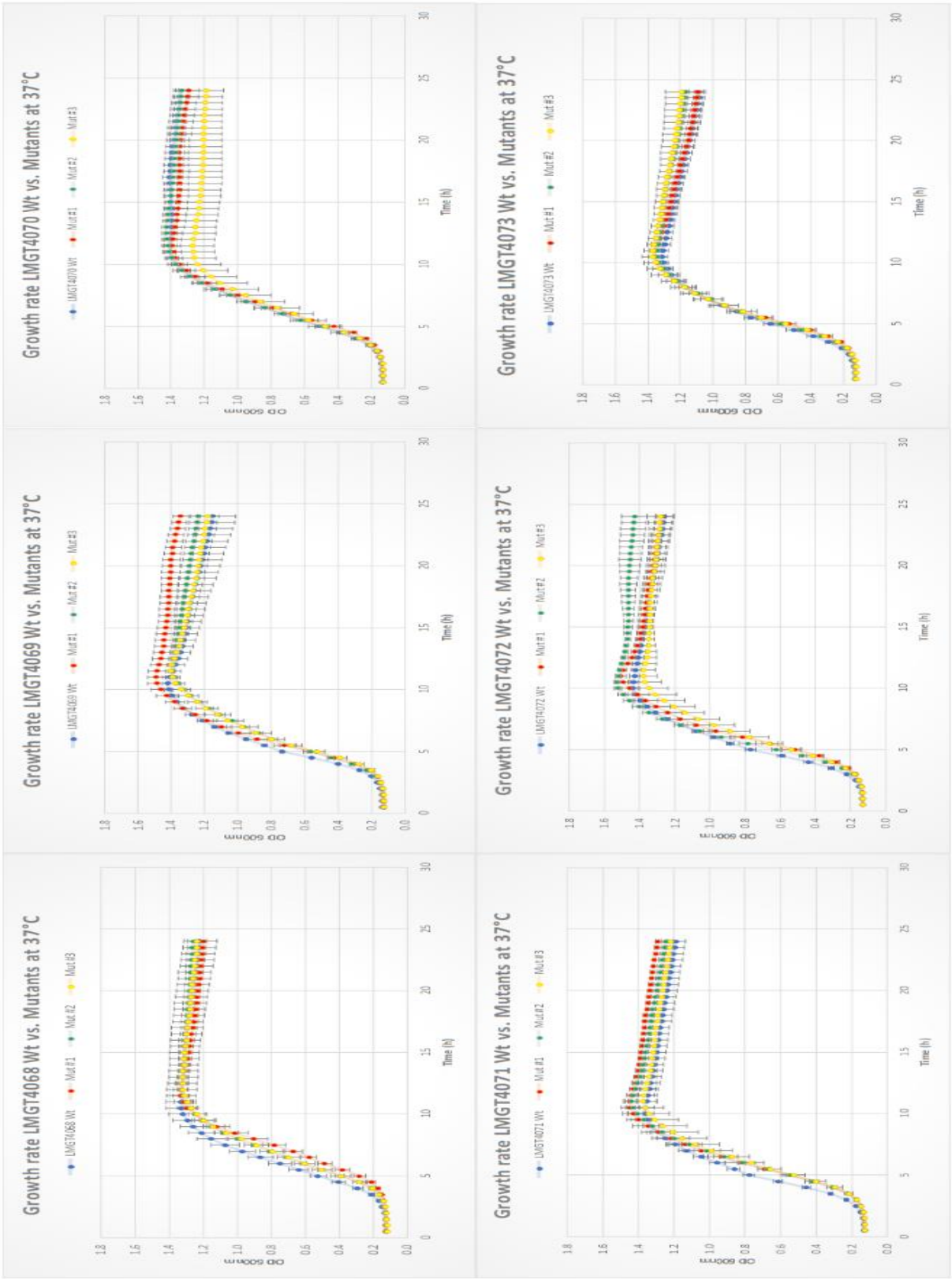

Figure 3.14. Graphical representation of average growth rate measured at OD_{600} every 30 minutes for 24 h including the six Indian *S. haemolyticus* wildtype strains (blue) with each of their respective EntK1EJ97-resistant mutants (red, yellow and green) in 200 µl BHI at 37°C.

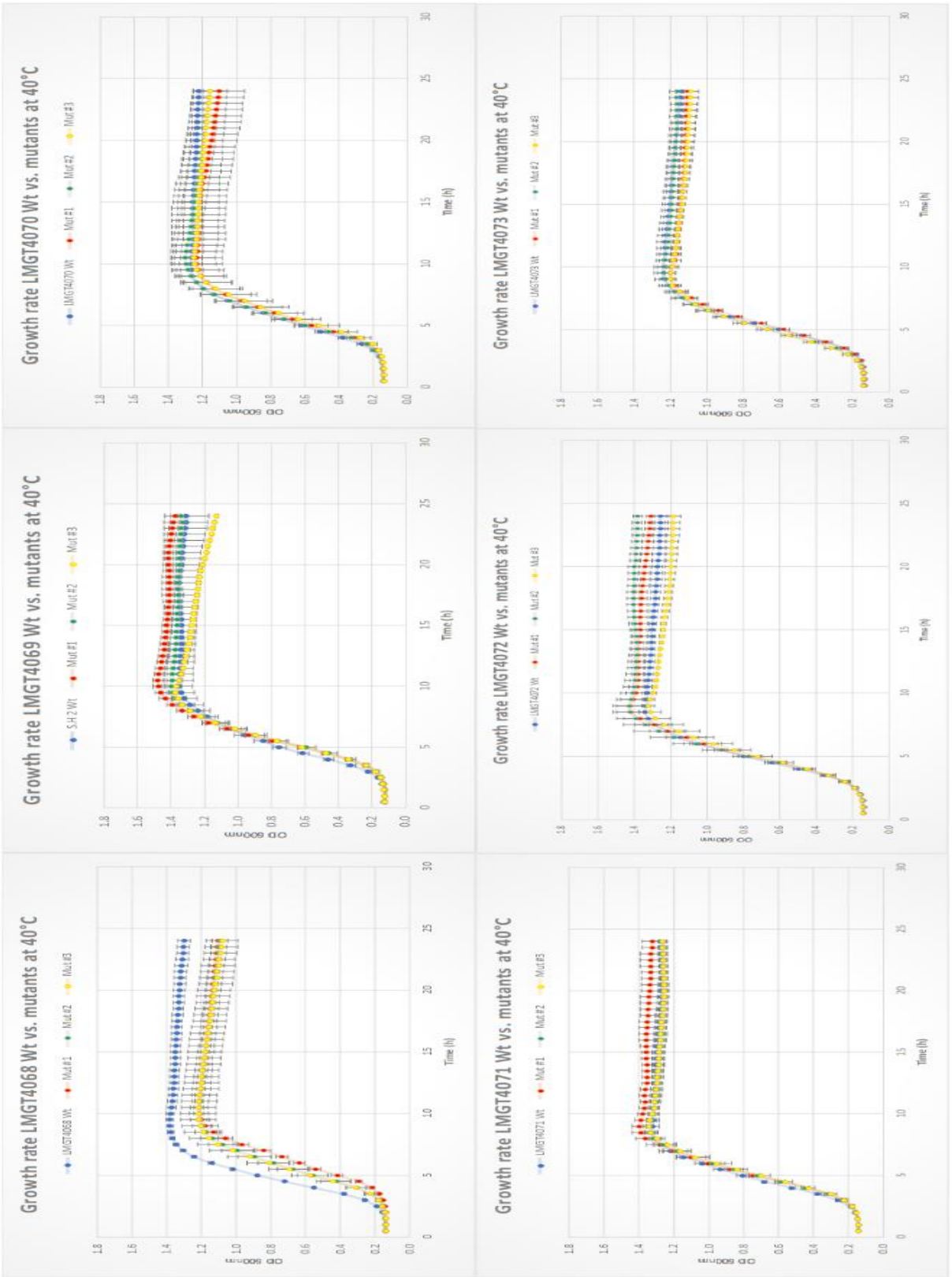

Figure 3.15. Graphical representation of average growth rate measured at OD_{600} every 30 minutes for 24 h including the six Indian *S. haemolyticus* wildtype strains (blue) with each of their respective EntK1EJ97-resistant mutants (red, yellow and green) in 200 µl BHI at 40°C.

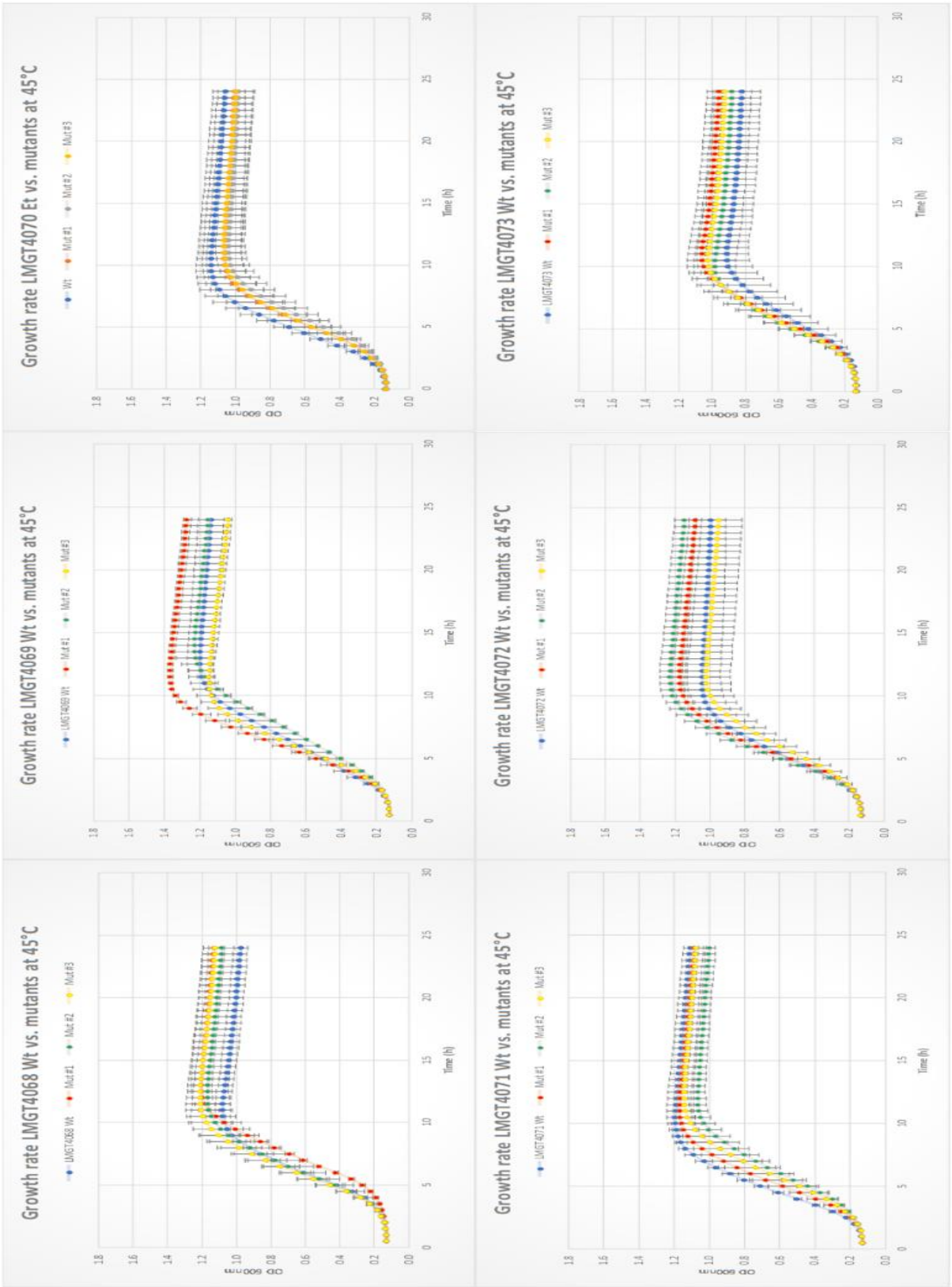

Figure 3.16. Graphical representation of average growth rate measured at OD_{600} every 30 minutes for 24 h including the six Indian *S. haemolyticus* wildtype strains (blue) with each of their respective EntK1EJ97-resistant mutants (red, yellow and green) in 200 µl BHI at 45°C.

3.11. *S. haemolyticus* wildtypes and their respective EntK1EJ97-resistant mutants show 100% sequence identity within their RseP receptor

Although the previous temperature-stress assay revealed no significant phenotypical difference in the wildtypes and their respective mutant's ability to adapt and grow under stressful temperature conditions, it was nevertheless decided to perform sequencing of the *rseP* gene in order to confirm that no mutations had occurred. For this purpose, it was decided to pick and sequence the *rseP* from one random Indian wildtype strain LMGT4068 and one random Norwegian wildtype strain LMGT4105 together with one random mutant from each of them.

Sequencing was performed on amplicons of mutants and subsequently aligned with respective wildtypes. Concentration and purity of the *rseP* PCR amplicons gave good enough yield when measured by NanoDrop and a small amplicon sample from both wildtypes and mutants were subsequently run on a 1% agarose gel electrophoresis.

As seen in **Figure 3.17**, the result gave good indication that the *rseP* at size ~ 1511 bp was successfully isolated by matching well with the 1500 bp fragment from the 1 kb ladder and the amplicons was ready to be sent for GATC sanger sequencing. Reads were assembled to contig which were translated to amino acids and analyzed by pairwise sequence alignment. As seen in **Figure 3.18** and **Figure 3.19,** there were no mutations at DNA-level of the RseP in neither of the resistant mutant isolates LMGT4068 and LMGT4105 compared to their respective wildtypes by showing 100% amino acid sequence identity.

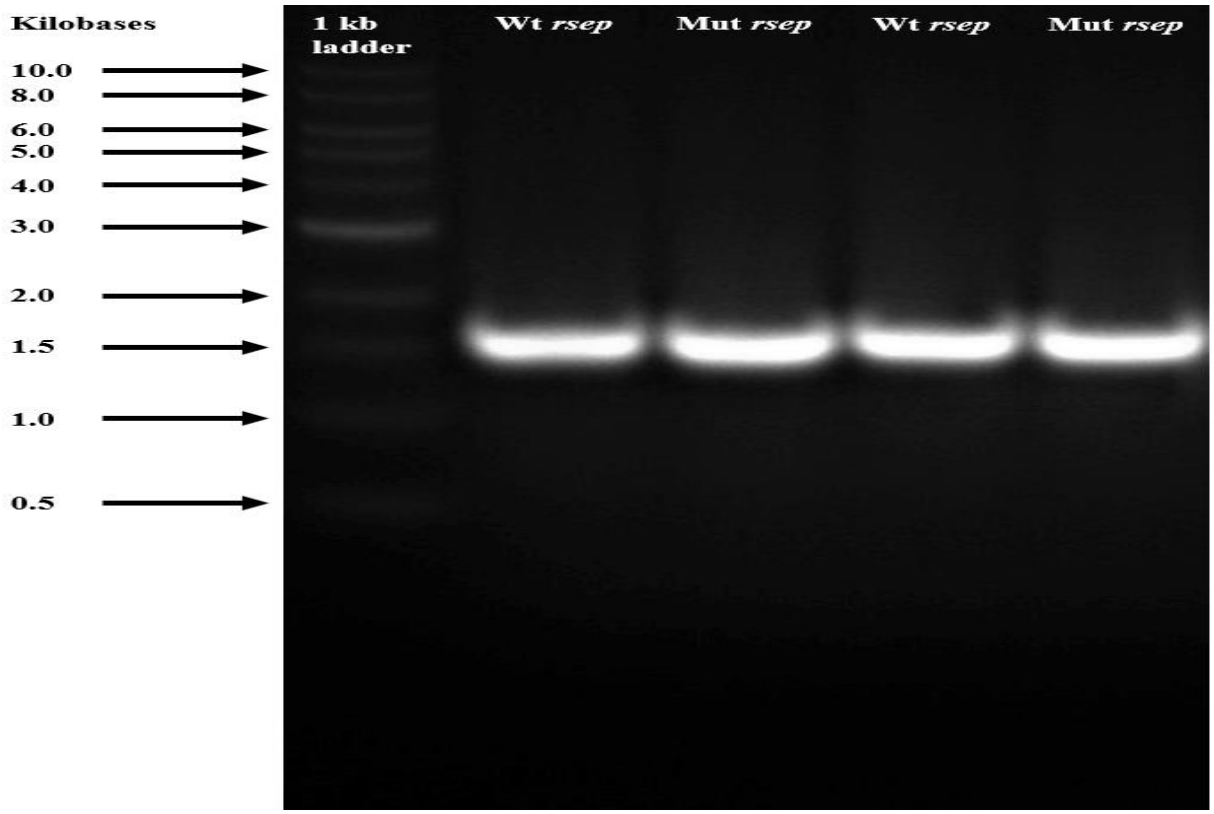

Figure 3.17. Amplified *rseP* DNA fragments from the two *S. haemolyticus* wildtype strains LMGT4068 and LMGT4105 together with one EntK1EJ97-resistant mutant from each strain. 10 µl PCR product was run on a 1.0% TAE gel at 90 V for 60 minutes. 1 kb ladder (NEB) was used as a marker.

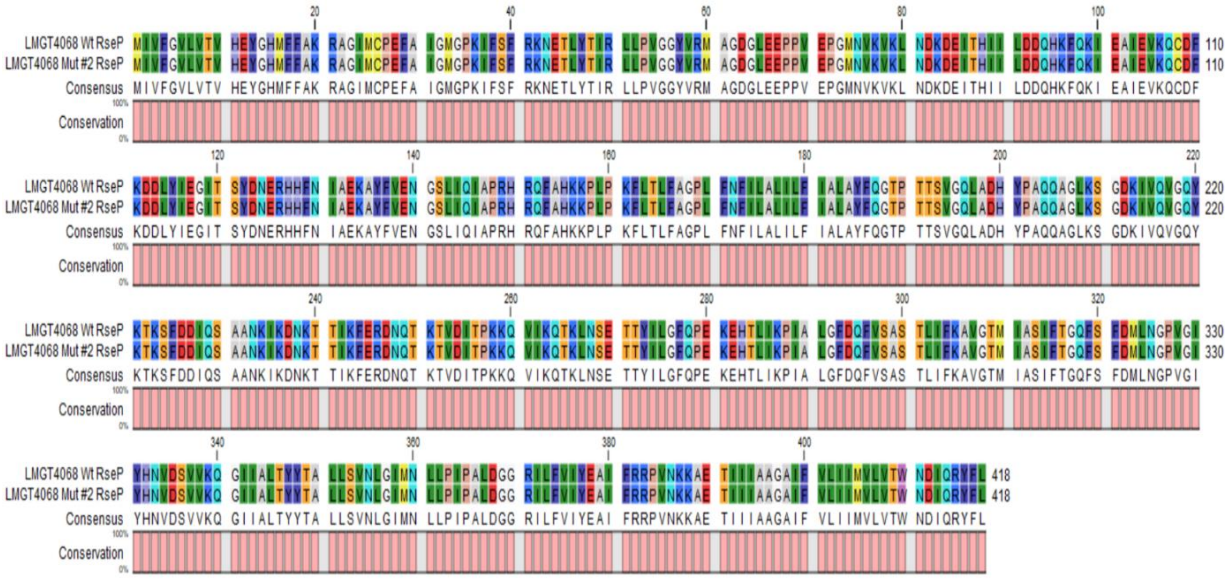

Figure 3.18. RseP amino acid sequence alignment of Indian *S. haemolyticus* wildtype strain LMGT4068 aligned with the amino acid sequence from one of its EntK1EJ97-resistant mutants using CLC sequence viewer 8.0 showing 100% sequence identity.

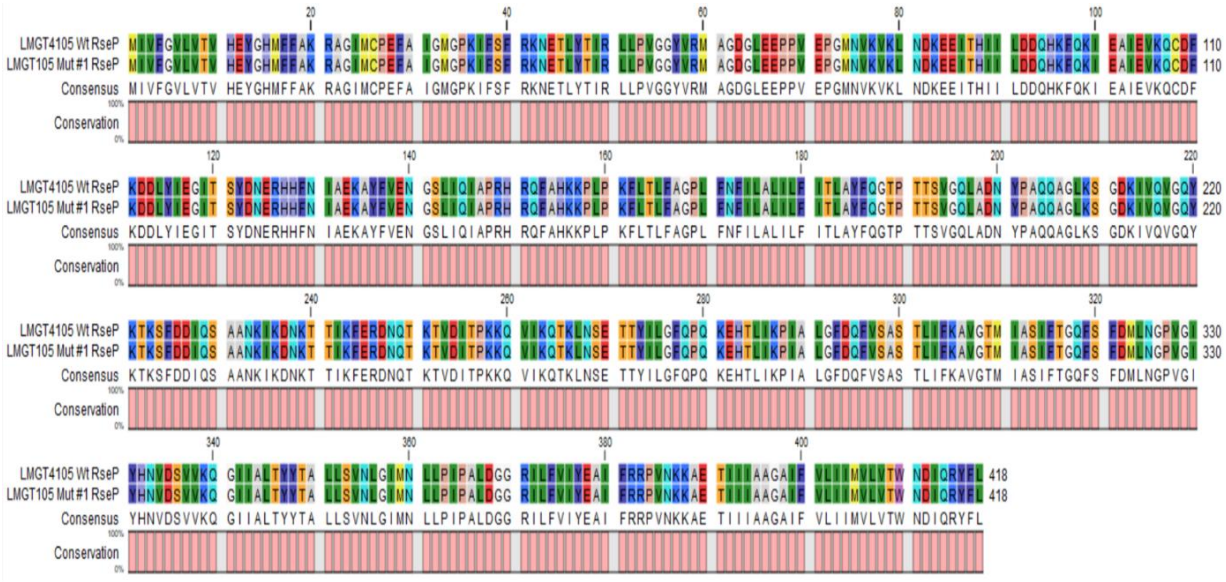

Figure 3.19. RseP amino acid sequence alignment of Norwegian *S. haemolyticus* wildtype strain LMGT4105 aligned with the amino acid sequence from one of its EntK1EJ97-resistant mutants using CLC sequence viewer 8.0 showing 100% sequence identity.

3.12. *S. haemolyticus* wildtypes and their respective EntK1EJ97-resistant mutants may have some morphological differences

Enhanced resistance against antimicrobials might be explained by reduced accessibility of the antimicrobial agent through the cell wall and/or cell membrane in order to reach its target [90]. As concluding the further investigation into the EntK1EJ97-resistant phenotype of the *S. haemolyticus* mutants in this study, morphological differences in the cell wall and cell membrane between the EntK1EJ97-sensitive wildtypes and their respective EntK1EJ97-resistant mutants was evaluated by transmission electron microscopy (TEM). Differences in antimicrobial susceptibility suggested that the resistant isolates might have visible variations on cell surface compared to their wildtypes [50][31]. For the premise of this investigation and in collaboration with Karolina Teresa Bartkiewicz (Master student, LMG) and her work on the Norwegian strains, it was decided to pick the two Norwegian *S. haemolyticus* wildtype strains LMGT4115 and LMGT4105 together with one EntK1EJ97-resistant mutant from each of them. In order to avoid a lot of cells dividing by binary fission when observed in the TEM, each specimen was picked for preparation when they were well within the stationary growth phase approximately 15 h after cultivation based on the growth rates in the temperature-stress assay.

As seen in **Figure 3.20** and **Figure 3.21**, obtained micrographs showed no notable variation in the cell membranes of the wildtypes and their respective mutants. However, the morphology and thickness of the resistant isolate's peptidoglycan composed cell wall differed visually compared to their wildtypes although no measurement of the thickness was performed in this experiment. The cell walls of the resistant isolates seemed to be much thicker and more rough-looking in their contour compared to the thinner and much smoother looking cell walls of their respective wildtypes. Also, it looked like the resistant isolates had a much higher content of the wall teichoic acid (WTA) and lipoteichoic acid (LTA), which are attached to the peptidoglycan layer and the cell membrane, respectively in Gram-positive bacteria.

However, whether these variations in cell wall morphology can be attributed to the observed EntK1EJ97-resistant phenotype of the *S. haemolyticus* mutants by perhaps preventing diffusion and binding access of EntK1EJ97 towards the RseP receptor or merely a consequence of sectioning or embedding error of the specimen is quite difficult to state at this point without any further investigation.

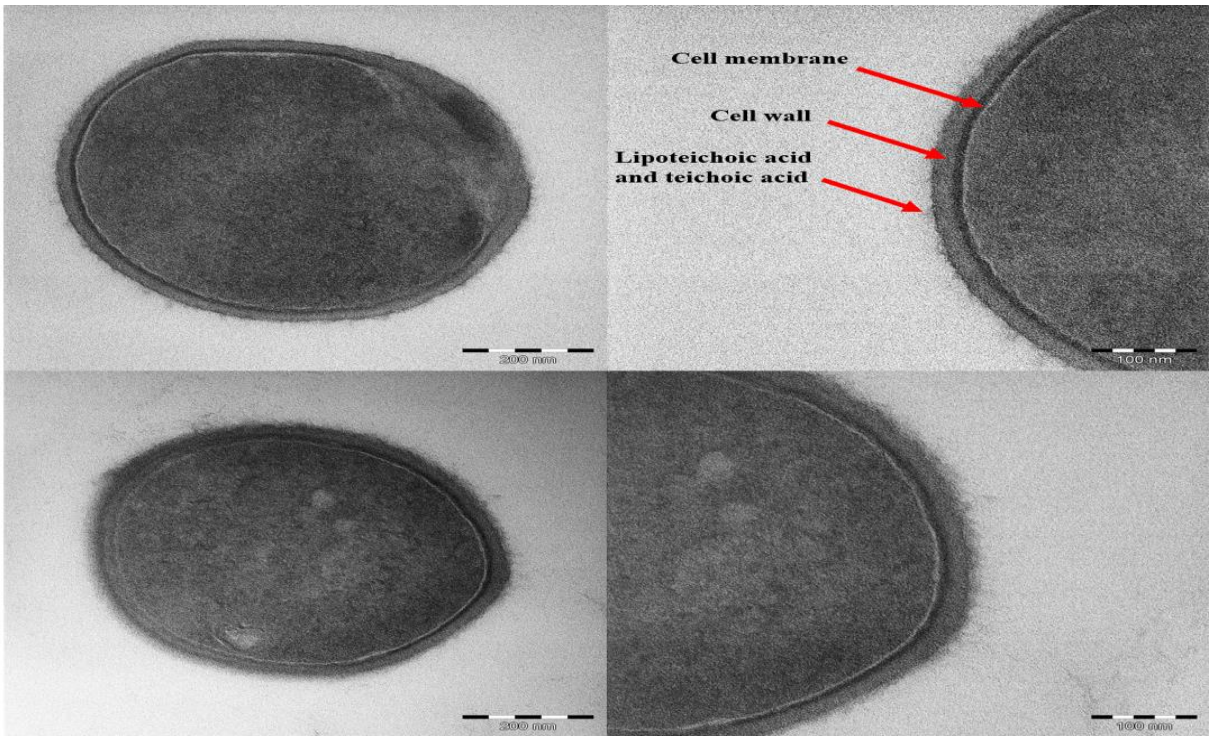

Figure 3.20. Transmission electron microscopy (TEM) micrographs showing the cell morphology of two random wildtype cells from the Norwegian *S. haemolyticus* strains LMGT4105 (top) and LMGT4115 (bottom) magnified 110Kx (left) and 180Kx (right).

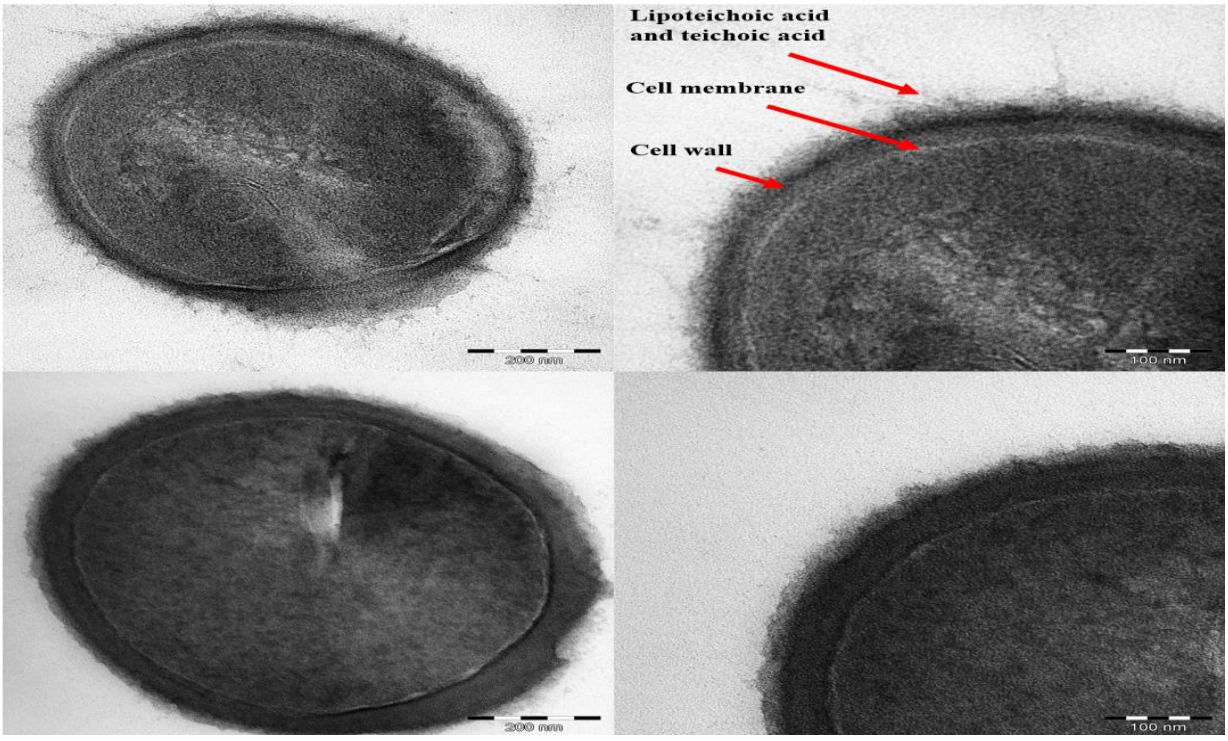

Figure 3.21. Transmission electron microscopy (TEM) micrographs showing the cell morphology of two random resistant mutant cells from the Norwegian *S. haemolyticus* strains LMGT4105 (top) and LMGT4115 (bottom) magnified 110Kx (left) and 180Kx (right).

4. Discussion

4.1. Main findings during this study

Some of the main findings during this study was that the hybrid bacteriocin EntK1EJ97 was active against the clinically important pathogen *S. haemolyticus* which was also found to be a strong *in vitro* biofilm producer under optimal conditions. However, EntK1EJ97-resistant planktonic and sessile *S. haemolyticus* mutant cells were generated. For the purpose of preventing the generation of EntK1EJ97-resistant mutants, various single and combinatorial bacteriocin treatments were applied with the bacteriocins EntK1EJ97, GarKS and MP1 in which the triple combinatorial treatment EntK1EJ97/GarKS/MP1 was the most effective treatment. Contrary to the single EntK1EJ97 treatments which was only effective after a short treatment (5 h), EntK1EJ97/GarKS/MP1 was also effective over extended time (24 – 48 h) without the generation of resistant mutants in both the planktonic cells and the sessile cells within the biofilms. In addition, the sessile cells within their biofilms was found to display a much higher degree of resistance towards the bacteriocin treatments compared to the planktonic cells. Several comparative studies were performed in order to perhaps explain the EntK1EJ97-resistant phenotype of the resistant mutants. However, temperature-stress assay and *rseP* sequencing indicated that the resistant mutants RseP receptor was functional in their regards to the cells ability to adapt and grow at stressful temperature conditions and that no mutations was found at the DNA-level of their RseP receptor. In concluding the further investigation into the resistant phenotype of the EntK1EJ97-resistant *S. haemolyticus* mutants, transmission electron microscopy (TEM) images were taken of both the EntK1EJ97-sensitive and resistant cells in which interesting morphological differences in the thickness and structure of the EntK1EJ97-resistant mutants cell walls compared to their EntK1EJ97-sensitive counterparts was observed.

4.2. The hybrid bacteriocin EntK1EJ97 was highly active against *S. haemolyticus*

The spectrum of activity assay performed with the bacteriocins EntK1, EntEJ97, the hybrid EntK1EJ97 and the opposite hybrid EntEJ97K1 showed that EntK1EJ97 had a relatively broad spectrum of activity resembling the activity of EntEJ97. EntK1EJ97 was especially active against clinically important species within the genera *Staphylococcus* and *Enterococcus*, displaying well-defined inhibition zones against *S. haemolyticus* although resistant mutant colonies were generated. The opposite hybrid bacteriocin EntEJ97K1 had the narrowest spectrum of activity and was mostly active against species within the *Lactobacillus* genera.

The observed similarities in the spectrum of activity displayed by EntEJ97 and EntK1EJ97 might suggest that the part of the LsbB-like bacteriocins which interacts with the RseP receptor is located at their C-terminal part which has been previously suggested [86]. The observed variation in spectrum of activity among the bacteriocins may in addition to the highly conserved residues of the KXXXGXXPWE motif, also indicate that other amino acid residues such as the last alanine amino acid residue located at the C-terminal might play an important role in interaction with the RseP receptor which has also been suggested [86].

The result of the comparative susceptibility of *S. haemolyticus* against various antibiotics often used to treat infections caused by staphylococci made it apparent that EntK1EJ97 was very active and an attractive alternative to conventional antibiotics. EntK1EJ97 gave some of the most consistent results for all the tested *S. haemolyticus* strains, although a large amount of EntK1EJ97 resistant mutant colonies were generated. The antimicrobial activity of erythromycin and ciprofloxacin displayed strain dependent activity while all the strains were resistant towards penicillin G. Bacteriocins have not yet been extensively used in clinical settings in the same degree as conventional antibiotics [31]. This might suggest that mutations within the gene encoding the specific bacteriocin target receptor or in genes that influence the expression or function of the receptor are less prevalent in clinically important pathogens, while mutations responsible for resistance against well-established antibiotics is already an issue in clinically isolated strains [19]. In this study however, resistance against EntK1EJ97 developed very quickly in all the S. haemolyticus strains.

4.3. Evaluation of *S. haemolyticus* as a strong biofilm producer

The *S. haemolyticus* strains from Norway and India, together with the Indian EntK1EJ97-resistant mutants, was determined to be strong *in vitro* biofilm producers when cultivated in plastic microtiter plates containing $TSB_{1\%GLU}$. Previous studies on *S. haemolyticus* biofilms has revealed that the ability to produce biofilms is significantly enhanced in TBS_{GLU} compared to TSB_{NaCl}, suggesting that the particular nutritional medium as well as sugar and salt concentration has a strong effect on biofilm production by *S. haemolyticus* and perhaps other pathogenic biofilm producing bacteria as well [48].

The classical microtiter plate biofilm assay with crystal violet staining of the cells is a common method for evaluating various bacterial species ability to produce biofilms *in vitro* [83]. Staphylococci has long been known for their strong ability to attach to plastic surfaces like these. However, less is known about their production of biofilms on other surfaces such as those on medical devices and *in vivo* on living tissue. Perhaps the classical microtiter plate assay for estimating biofilms in addition to medium known to enhance biofilm production might give an over-estimation of their biofilm producing abilities on other surfaces such as on medical devices which soon after insertion is covered by host matrix proteins, various biological molecules and gives access to different nutritional factors in the environment [83].

4.4. The effectiveness of combinatorial bacteriocin treatments against planktonic and sessile *S. haemolyticus* cells

The comparative study with single and combinatorial bacteriocin treatments against both the planktonic and the sessile cells within their biofilms showed that the single bacteriocin treatments was most effective after a short incubation and when the incubation was prolonged, their average MIC_{50} increased considerably. However, their effectiveness was significantly increased by applying them in various combinations in which the triple combinatorial EntK1EJ97/GarKS/MP1 bacteriocin treatment was most effective and displayed the strongest synergistic effect against the strains. This treatment decreased the inter-strain MIC_{50} variability, reduced the concentration of each bacteriocin needed to

inhibit bacterial growth and successfully prevented the generation of EntK1EJ97-resistant *S. haemolyticus* mutants.

Several studies previously done on combinatorial antimicrobial treatments confirms the outcome of these results. In contrast to treatments with single antimicrobials, combinations of different antimicrobials with their own specific mechanism of action mainly leads to synergism or antagonism. The synergistic response has a much greater effect than the single antimicrobial treatment would and more than just an additive effect. Combinatorial antimicrobial treatments have also been shown to greatly reduce the probability of the target bacteria evolving resistance due to the different targets involved and since it is considerably more unlikely that pathogenic bacteria simultaneously develop resistance against multiple antimicrobial agents [117][74]. Studies have revealed the benefits and advantageous of combinatorial treatments which includes effective inhibition of multidrug-resistant (MDR) and biofilm producing pathogenic bacteria such as *S. aureus* (MRSA), reducing the possibility of resistance development, reduction of single doses and decreasing the chance of potential off-target side effects [69]. However, possible pitfalls to this approach may include drug-drug interactions and inhibition of harmless or beneficial commensal bacterial communities and it is of utmost importance to take this into consideration when developing the combinatorial treatment with cocktails of different antimicrobial agents [116].

4.5. Evaluation of the increased degree of bacteriocin resistance in sessile *S. haemolyticus* cells within biofilms

The single EntK1EJ97 and the triple combinatorial EntK1EJ97/GarKS/MP1 bacteriocin treatments against the sessile cells within their biofilms revealed a much higher degree of resistance against these treatments compared to their planktonic cells. Biofilms have long been known to dramatically increase the degree of resistance against antimicrobials and even though the defense mechanism of biofilms against antimicrobials are not yet entirely understood, several possible mechanisms have been suggested to contribute to the increased biofilm resistance [62][95]. In case of this study, one or more of these suggested

mechanisms may attribute to the increased resistance observed for the *S. haemolyticus* biofilms.

Some of the possible explanation for the increased resistance observed may first and foremost be the structure of the biofilm such as its 3-dimensional shape and thickness together with the physical nature of the biofilm producing bacteria which has been shown to confer an inherent resistance against antimicrobials [39]. Other suggested mechanisms include an altered gene expression in biofilm-specific resistance genes compared to the planktonic cells such as those encoding efflux pumps or antimicrobial destroying enzymes such as proteases, an altered microenvironment inside the biofilm matrix such as pH-levels which might cause the antimicrobial to be less effective, slow growth and metabolism of the cells within the biofilm [104][105] and prevention of the antimicrobial from reaching its target by limited diffusion through the biofilm or repulsion. These are all examples of possible reasons which might limit the effectiveness of the antimicrobial against the biofilm [62].

4.6. Evaluation of the EntK1EJ97-resistant phenotype of the *S. haemolyticus* mutants

The EntK1EJ97-resistant *S. haemolyticus* mutants were observed throughout this study and several comparative experiments were performed on both the EntK1EJ97-sensitive wildtypes and their respective resistant mutants. The MIC assay against the planktonic cells and the BOAT against the sessile cells within the biofilms made it particularly obvious that there was a difference in the susceptibility for EntK1EJ97 between the wildtypes and their resistant mutants. The growth of the wildtypes was effectively inhibited after a short incubation with the EntK1EJ97 treatment, while EntK1EJ97-resistant mutants was generated after a prolonged incubation.

Several different factors may be responsible for the observed EntK1EJ97-resistant phenotype of the mutants. One possible reason for bacteriocin resistance may be that the cellular receptor, in this case the RseP receptor, that the bacteriocin interacts with have mutated to such degree that the affinity to the bacteriocin is reduced or completely lost [42]. However, this reason may be eliminated for the observed EntK1EJ97-resistance since both the

temperature-stress assay and the *rseP* sequencing of the wildtypes and their respective resistant mutants showed that there was no significant difference in their ability to adapt and grow at increasing temperature conditions and that there had not occurred any mutations at the DNA-level of their RseP receptor.

A second possible reason for bacteriocin resistance could be that both the composition of the cell wall and the cell membrane may hinder diffusion of the bacteriocin and making it physically unable to reach its target site [42]. Possible resistance mechanisms for those bacteriocins that primarily targets the cell membrane have been identified. For example, previous studies on *S. aureus* with intermediate resistance against vancomycin have indicated that enhanced resistance to lipid II-targeting lantibiotics could emerge as a consequence of reduced accessibility to the cell membrane receptor [90].

Observation of the *S. haemolyticus* wildtypes and their respective EntK1EJ97-resistant mutants by applying transmission electron microscopy (TEM), indicated that there perhaps were some interesting morphological differences in the composition and structure of their cell walls. It seemed that the cell walls of the resistant mutants were much thicker and had a higher content of the wall teichoic acid (WTA) and lipoteichoic acid (LTA) protruding from the surface of the cell walls compared to their wildtypes. However, no comparative and statistical measurements of the thickness of the cell walls was performed in this study so whether these morphological differences may be the cause of the observed resistant phenotype of the mutants or perhaps due to some errors in the processing of the specimen are just speculations at this point and more research into this matter needs to be done in order to confirm or disconfirm these findings.

It has also been suggested that the presence of proteases may reduce or proteolytically cleave the bacteriocin and thereby reduce its effectiveness against the target cell. Resistance against bacteriocins may also be obtained by the expression of dedicated immunity genes. For example, the Gram-positive bacteriocin-producing lactic acid bacteria (LAB) are resistant to their own bacteriocins because they express cognate immunity genes, which are often co-transcribed with the structural gene for the bacteriocin which they produce [79].

Bacteriocin gene clusters also contain multiple genes whose products have not yet been characterized and which thus have unknown functions. Such genes may play important roles in unknown resistance mechanisms [42]. Reduction or loss of gene expression of cell membrane-associated receptors might also be responsible for the observed enhanced resistant phenotype against some bacteriocins [63].

Further research on the potential mechanisms behind bacteriocin resistance is of utmost importance for minimizing the emergence of resistance when these antimicrobial peptides are to be applied in clinical settings.

4.7. Final remarks and suggestions for further research

Unfortunately, due to the pandemic outbreak of COVID-19 and the laboratory restrictions and regulations that followed, it was not possible to replicate all the experiments performed in this study in three independent experiments.

However, this study revealed that the newly designed and bioengineered hybrid bacteriocin EntK1EJ97 could be considered as an alternative for conventional antibiotics against both planktonic cells and biofilm producing sessile cells of the clinically important pathogen *S. haemolyticus*. However, EntK1EJ97-resistant mutants were generated. The EntK1EJ97-resistant mutants were effectively inhibited by applying combinatorial bacteriocin treatments of which the triple combinatorial EntK1EJ97/GarKS/MP1 treatment was most effective.

Further investigation into the effectiveness of this combinatorial treatment against the *S. haemolyticus* biofilms could be done by evaluating its effect on the sessile cells within the biofilms and quantitatively analyzing the cells vitality through Live/Dead fluorescence microscopy [60] and by performing comparative studies on treated/untreated biofilms with scanning electron microscopy (SEM) in order to observe any differences in the appearance and possible damages to the structure of the biofilms and damages to the sessile cells within them. In addition, live animal models could be locally and systemically infected with *S. haemolyticus* in order to apply the triple combinatorial bacteriocin treatment and assess the minimum inhibitory concentration needed to completely treat the infections.

The biofilm assay revealed that all the *S. haemolyticus* strains and their tested EntK1EJ97-resistant mutants were strong *in vitro* biofilm producers within the wells of the microtiter plates with $TSB_{1\%GLU}$. However, the classical microtiter plate assay for evaluating bacterial biofilm production under optimal conditions might not give an accurate depiction of their abilities to produce biofilms on other abiotic or biotic surfaces and under other nutritional and environmental conditions. Thus, biofilm assays should perhaps be accompanied with more elaborate *in vitro* and *in vivo* methods for more accurate estimation of biofilm production, such as flow cells, confocal laser scanning microscopy and animal models of biofilm-associated infection [83].

In addition to the use of combinatorial treatments to prevent the generation of resistant mutants, EntK1EJ97-resistance might also be prevented by further research and knowledge of the mechanism of interaction between the RseP receptor and the bacteriocin, possibly through the use of optical methods which can monitor protein interactions such as fluorescence resonance energy transfer (FRET). By labeling EntK1EJ97 with a fluorescent tag and the RseP receptor with another fluorescent tag such that the emission spectrum of one fluorescent tag overlaps with the other, the energy of the absorbed light could be transferred from one fluorescent protein to the other which might give more knowledge about the EntK1EJ97-RseP interaction [16]. However, a much more detailed structural investigation of the interaction at an atomic level could be provided by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, although these methods are expensive, much more difficult and quite laborious to perform [107]. The resistance could perhaps also be overcome by slight structural modifications of the bacteriocin by the application of the genetic modification technology CRISPR, perhaps without altering its effectiveness against the *S. haemolyticus* in general [74].

Another goal of this study was to investigate the mechanism of resistance in the isolated EntK1EJ97-resistant mutants. However, temperature-stress assay and sequencing of the *rseP* gene indicated that the RseP was functional and that no mutation had occurred at the DNA-level for the RseP receptor which it interacts with. Although the mutants was confirmed to contain the intact *rseP* gene, it would be interesting to perform western blotting of the RseP receptor on both the wildtypes and their mutants to investigate whether any altered expression of the *rseP* gene and the subsequent amount of the RseP receptor may explain the observed resistance against EntK1EJ97. It would also be interesting to perform comparative and statistical measurements of the thickness of the cell walls of the wildtypes and their respective EntK1EJ97-resistant mutants.

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Appendix

A1: Complete collection of bacteriocin spectrum of activity for 51 strains-different genera. Scoring system: 0 = no inhibition; 1 = Unclear zone; 2 = small zone (< 1 cm); 3 = big zone (≥ 1 cm), "*" indicate zones with resistant mutant colonies.

	[EntK1] (mg/ml)			[EntEJ97] (mg/ml)			[EntK1EJ97] (mg/ml)			[EntEJ97K1] (mg/ml)			[Nisin Z] (mg/ml)		
Isolate	0.04	0.2	1.0	0.04	0.2	1.0	0.04	0.2	1.0	0.04	0.2	1.0	0.04	0.2	1.0
Staphylococcus homonis (n=1)	1	1	2	1	2	3	1	2*	3*	0	1	1	1	2	2
Staphylococcus haemolyticus (n=1)	0	0	1	0	0	2	1	2*	3*	0	0	1	2	2	3*
Staphylococcus epidermidis (n=1)	0	0	1	0	1	1	0	1	2*	0	0	0	2	2	3
Staphylococcus aureus (n=6)	0	0	1	0	1	1	0	0	1	0	0	0	1	2*	2
Staphylococcus arlettae (n=1)	0	0	0	0	0	1	0	0	0	0	0	1	1	2	2
Staphylococcus simulans (n=1)	0	0	0	0	0	0	0	0	0	0	0	0	1	2	2
Bacillus cereus (n=3)	0	0	1	0	0	1	0	0	1	0	0	0	2	2	2
Listeria ivanovii (n=1)	0	0	1	0	1	1	0	1	1	0	0	1	2	2	3
Listeria innocua (n=2)	0	1	2*	1	1	1	1	2*	2*	0	0	2*	2*	2*	3*
Listeria monocytogenes (n=3)	0	0	1	0	0	0	0	0	1	0	0	0	2*	2	2
Carnobacterium pisciola (n=1)	1	1	1	0	1	1	1	1	1	0	0	0	2*	2	2
Carnobacterium divergens (n=1)	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2
Enterococcus faecalis (n=5)	0	1	1	1	2	3*	1	2	3	0	1	1	2	2	2
Enterococcus avium (n=1)	0	0	1	0	1	1	1	2*	3*	0	0	1	1	1	2
Enterococcus faecium (n=4)	1	2*	2	1	2	3	1	2*	3*	0	1	1	1	2	2
Lactobacillus salivarius (n=1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lactobacillus plantarum (n=2)	0	0	1	0	1	2	0	0	0	0	1	1	2*	2	3
Lactobacillus curvatus (n=1)	2*	2	3	2*	3	3	0	1	2	2*	2	3	2	3	3
Lactobacillus sakei (n=2)	0	0	2*	0	0	0	0	0	0	1	2*	2*	2	2	3
Lactococcus garvieae (n=1)	1	2*	2*	1	1	1	1	2*	2*	1	1	2*	2	3*	3
Lactococcus lactis (n=2)	0	1	1	1	1	1	1	2*	1	0	0	1	2	3*	3*
Streptococcus thermophilus (n=1)	0	0	1	0	0	0	0	1	3*	0	0	0	2	2	3
Streptococcus dysgalactiae (n=1)	0	1	1	0	1	1	0	1	1	0	0	0	2*	3	3
Streptococcus uberis (n=2)	0	0	1	0	0	0	0	1	2*	0	0	0	2	2	3
Leuconostoc gelidium (n=1)	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2
Escherichia coli (n=2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

A2: Average minimum inhibitory concentration (MIC_{50}) in µg/ml after 5 h exposure to a 1:1 dilution series with single and combinatorial bacteriocin treatments with the bacteriocins EntK1EJ97, GarKS and MP1 against planktonic cells of the six Indian *S. haemolyticus* strains.

Treatment	MIC_{50} [EntK1EJ97] (µg/ml)	MIC_{50} [GarKS] (µg/ml)	MIC_{50} [MP1] (µg/ml)	MIC_{50} [EntK1EJ97/GarKS] (µg/ml)	MIC_{50} [EntK1EJ97/MP1] (µg/ml)	MIC_{50} [GarKS/MP1] (µg/ml)	MIC_{50} [EntK1EJ97/GarKS/MP1] (µg/ml)
Duration	5 h	5 h	5 h	5 h	5 h	5 h	5 h
Strain							
LMGT4068	0.78	≤12.50	0.02	≤1.56/1.56	0.20/0.02	0.20/0.02	≤0.39/0.39/0.04
LMGT4069	0.78	≤6.25	≤0.16	≤1.56/1.56	≤1.56/0.16	≤3.13/0.31	≤0.78/0.78/0.08
LMGT4070	0.78	≤12.50	0.02	≤0.78/0.78	≤0.39/0.04	0.20/0.02	≤0.39/0.39/0.04
LMGT4071	0.78	12.50	≤0.08	≤3.13/3.13	≤0.78/0.08	≤0.78/0.08	≤0.78/0.78/0.08
LMGT4072	0.78	≤25.00	0.04	≤1.56/1.56	≤0.39/0.04	≤0.78/0.08	≤0.78/0.78/0.08
LMGT4073	0.78	12.50	≤0.04	≤3.13/3.13	0.20/0.02	0.20/0.02	≤0.39/0.39/0.04

A3: Average minimum inhibitory concentration (MIC_{50}) in µg/ml after 24 h exposure to a 1:1 dilution series with single and combinatorial bacteriocin treatments with the bacteriocins EntK1EJ97, GarKS and MP1 against planktonic cells of the six Indian *S. haemolyticus* strains. "R" indicates generation of resistant mutants and "NO" indicates that the MIC_{50} exceeded the highest concentration in the dilution series and could not be determined.

Treatment	MIC_{50} [EntK1EJ97] (µg/ml)	MIC_{50} [GarKS] (µg/ml)	MIC_{50} [MP1] (µg/ml)	MIC_{50} [EntK1EJ97/GarKS] (µg/ml)	MIC_{50} [EntK1EJ97/MP1] (µg/ml)	MIC_{50} [GarKS/MP1] (µg/ml)	MIC_{50} [EntK1EJ97/GarKS/MP1] (µg/ml)
Duration	24 h	24 h	24 h	24 h	24 h	24 h	24 h
Strain							
LMGT4068	R	≤25.00	≤0.04	3.13/3.13	0.78/0.08	≤0.39/0.04	0.78/0.78/0.08
LMGT4069	0.78	≤25.00	NO	≤6.25/6.25	≤25.00/2.50	≤50.00/5.00	≤6.25/6.25/0.63
LMGT4070	R	≤50.00	≤0.63	≤6.25/6.25	≤3.13/0.31	≤6.25/0.63	≤3.13/3.13/0.31
LMGT4071	R	≤100.00	10.00	≤12.50/12.50	≤1.56/0.16	6.25/0.63	≤3.13/3.13/0.31
LMGT4072	R	≤50.00	≤0.31	≤12.50/12.50	≤1.56/0.16	≤3.13/0.31	1.56/1.56/0.16
LMGT4073	R	≤100.00	0.04	6.25/6.25	≤0.39/0.04	≤0.78/0.08	≤0.78/0.78/0.08

A4: Average minimum inhibitory concentration (MIC_{50}) in µg/ml after 48 h exposure to a 1:1 dilution series with single and combinatorial bacteriocin treatments with the bacteriocins EntK1EJ97, GarKS and MP1 against planktonic cells of the six Indian *S. haemolyticus* strains. "R" indicates generation of resistant mutants and "NO" indicates that the MIC_{50} exceeded the highest concentration in the dilution series and could not be determined.

Treatment	MIC_{50} [EntK1EJ97] (µg/ml)	MIC_{50} [GarKS] (µg/ml)	MIC_{50} [MP1] (µg/ml)	MIC_{50} [EntK1EJ97/GarKS] (µg/ml)	MIC_{50} [EntK1EJ97/MP1] (µg/ml)	MIC_{50} [GarKS/MP1] (µg/ml)	MIC_{50} [EntK1EJ97/GarKS/MP1] (µg/ml)
Duration	48 h	48 h	48 h	48 h	48 h	48 h	48 h
Strain							
LMGT4068	R	25.00	0.08	≤6.25/6.25	≤0.78/0.08	≤0.78/0.08	≤3.13/3.13/0.31
LMGT4069	R	≤50.00	NO	≤12.50/12.50	≤50.00/5.00	50.00/5.00	≤6.25/6.25/0.63
LMGT4070	R	≤100.00	1.25	6.25/6.25	≤6.25/0.63	6.25/0.63	≤6.25/6.25/0.63
LMGT4071	R	≤100.00	10.00	≤25.00/25.00	≤3.13/0.31	≤12.50/1.25	≤6.25/6.25/0.63
LMGT4072	R	≤100.00	≤0.63	≤25.00/25.00	1.56/0.16	3.13/0.31	≤6.25/6.25/0.63
LMGT4073	R	≤100.00	0.16	12.50/12.50	≤1.56/0.16	≤1.56/0.16	≤1.56/1.56/0.16

A5: Average minimum inhibitory concentration (MIC_{50}) in µg/ml after 5 h and 24 h exposure to a 1:1 dilution series with bacteriocin EntK1EJ97 against sessile cells within biofilms of the six Indian *S. haemolyticus* strains, the Norwegian *S. haemolyticus* strain LMGT4115, the *S. aureus* (MRSA) strain USA300 and the *S arlettae* strain LMGT4059.

Treatment	**MIC_{50} [EntK1EJ97] (µg/ml)**	**MIC_{50} [EntK1EJ97] (µg/ml)**
Duration	5 h	24 h
Strain		
LMGT4068	≥5.00	>160.00
LMGT4069	≥80.00	>160.00
LMGT4070	≥20.00	>160.00
LMGT4071	≥40.00	>160.00
LMGT4072	≥20.00	>160.00
LMGT4073	≥10.00	>160.00
LMGT4115	≥10.00	>160.00
USA300	>160.00	>160.00
LMGT4059	>160.00	>160.00

A6: Average minimum inhibitory concentration (MIC_{50}) in µg/ml after 5-, 24-, and 48 h exposure time to a 1:1 dilution series with the triple combinatorial EntK1EJ97/GarKS/MP1 bacteriocin treatment against sessile cells within biofilms of the six Indian *S. haemolyticus* strains and the seven Norwegian *S. haemolyticus* strains.

Treatment	**MIC_{50} [EntK1EJ97/GarKS/MP1] (µg/ml)**	**MIC_{50} [EntK1EJ97/GarKS/MP1] (µg/ml)**	**MIC_{50} [EntK1EJ97/GarKS/MP1] (µg/ml)**
Duration	5 h	24 h	48 h
Strain			
LMGT4068	<4.88/4.88/0.49	<4.88/4.88/0.49	≥9.77/9.77/0.98
LMGT4069	≥19.53/19.53/1.95	≥39.06/39.06/3.91	≥78.13/78.13/7.81
LMGT4070	<4.88/4.88/0.49	≥9.77/9.77/0.98	≥19.53/19.53/1.95
LMGT4071	≥4.88/4.88/0.49	<4.88/4.88/0.49	<4.88/4.88/0.49
LMGT4072	<4.88/4.88/0.49	<4.88/4.88/0.49	<4.88/4.88/0.49
LMGT4073	<4.88/4.88/0.49	<4.88/4.88/0.49	<4.88/4.88/0.49
LMGT4097	≥9.77/9.77/0.98	≥9.77/9.77/0.98	≥19.53/19.53/1.95
LMGT4103	≥9.77/9.77/0.98	≥19.53/19.53/1.95	≥39.06/39.06/3.91
LMGT4105	≥9.77/9.77/0.98	<4.88/4.88/0.49	<4.88/4.88/0.49
LMGT4106	<4.88/4.88/0.49	<4.88/4.88/0.49	<4.88/4.88/0.49
LMGT4113	≥4.88/4.88/0.49	≥4.88/4.88/0.49	≥39.06/39.06/3.91
LMGT4115	≥9.77/9.77/0.98	≥9.77/9.77/0.98	≥19.53/19.53/1.95
LMGT4132	<4.88/4.88/0.49	>19.53/19.53/1.95	≥39.06/39.06/3.91

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