The isolation and assessment of the antimicrobial activity of bacteriolytic and bactericidal endolysins of *Enterobacteriaceae* infecting bacteriophages

By

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our abilities and approved the final document for submission to the Faculty of Health Science for examination by the University appointed Examiners.

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PUBLICATIONS FROM THIS THESIS

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ABSTRACT

Background

Pathogenic bacteria pose a significant threat to the health and well-being of vulnerable populations, including newborns and young children, individuals with compromised immune systems as well as those hospitalized or recovering from surgery. The worldwide burden of communicable diseases driven by pathogenic bacteria continues to be a pressing global health issue, with a substantial impact on mortality rates. The presence of antimicrobial resistance further complicates the treatment of bacterial infections. The changing patterns of antimicrobial resistance have greatly decreased the survival rate of individuals with bacterial infections. Irresponsible stewardship of antibiotics, coupled with a lack of knowledge on the use of antibiotics by the public has further fueled the development of antimicrobial resistance. Furthermore, the World Health Organization's Review on Antimicrobial Resistance states that, if the current trend in antimicrobial resistance continues, it can lead to a staggering 10 million deaths per year by 2050. Given that antimicrobial resistance is a globally pressing issue, new and innovative strategies and treatments are required to combat the burden posed by antimicrobial resistance. Bacteriophage lytic enzymes offer a promising solution to curb the issue of antimicrobial resistance.

Aim

The primary aim of the present study was to evaluate the bacteriolytic and bactericidal activity of bacteriophage proteins against *Enterobacteriaceae* and *Staphylococcus aureus.*

Methodology

In the present study, the *Escherichia* T4 Bacteriophage along with its host, *Escherichia coli* (DSM No 613, ATCC 11303, respectively; Leibniz Institute DSMZ) formed the basis of the work. After reconstitution in nutrient broth, the *E. coli* was cultured on nutrient agar (1.5%) in a 90 mm petri dish, to which nutrient agar (0.6%) containing the T4 bacteriophage was added to enumerate the bacteriophage. *Escherichia coli* and bacteriophage T4 were then co-cultivated in one litre of nutrient broth and the

salting out method and column chromatography was used to isolate the biologically active proteins from the nutrient broth and proteomic analysis was performed to confirm the presence of the endolysin protein. Liposomes were created using the thin film hydration method and 1 ml of purified protein in phosphate buffer was encapsulated within the liposomes. Liposome formation was confirmed by Transmission electron microscopy and successful encapsulation of the protein was confirmed by breaking open 200 µl of liposomes and performing the Bradford protein quantification assay. Antimicrobial activity of T4 proteins and T4 protein loaded liposomes was evaluated using either a spot lytic assay or microdilution bioassay. In the latter, 100 µl of the bacteriophage protein solution (maintained in PBS) or 50 µl of the liposomal protein were serially diluted two-fold with 100 µl and 50 µl of sterile distilled water, respectively, yielding concentrations ranging from 205.68 µg/ml to 3.22 µg/ml. These samples were tested against three Gram-negative organisms, *Proteus mirabilis*, *Klebsiella pneumoniae, Escherichia coli* and two Gram-positive organisms *Staphylococcus aureus* and *Enterococcus faecalis.*

Results

Bacteriophage T4 proteins demonstrated varying levels of effectiveness in inhibiting the growth of the bacteria assessed in this study. For instance, in a microdilution assay, while the protein was able to inhibit the growth of *Staphylococcus aureus* at a concentration of 12.87 µg/ml and *Escherichia coli* at 43.70 µg/ml, it was incapable of influencing the growth of *Enterococcus faecalis*, *Proteus mirabilis* and *Klebsiella pneumoniae* at the highest concentration tested (205.68 µg/ml). Notably, in every instance, the liposomes loaded with T4 protein were more effective at inhibiting the growth of the bacteria. Molecular interactions were determined through protein-protein modeling that revealed that the endolysin protein from bacteriophage T4 was capable of binding with strong affinity to penicillin binding proteins from *S. aureus*.

Conclusion

The results of this study demonstrated the capability of T4 protein loaded liposomes to interact with and inhibit the growth of important bacteria linked to the BPPL as

identified by the WHO, providing a pathway for the development of novel drugs aimed at combating these devastating bacteria.

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LIST OF ABBREVIATIONS

ABR	Antibacterial resistance
ADP	Adenosine diphosphate
AGC	Automatic gain control
AMR	Antimicrobial resistance
ATP	Adenosine triphosphate
BPPL	Bacterial priority pathogens list
CFU	Colony forming unit
dCMP	Deoxycytidine monophosphate
dCTP	Deoxycytidine triphosphate
DNA	Deoxyribonucleic acid
dNMP	Deoxyribonucleoside monophosphate
dsDNA	Double strand deoxyribose nucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HCD	higher energy collisional dissociation
HCL	Hydrochloric acid
HILIC	Hydrophilic interaction chromatography
Hr	Hour
IV	Intravenous
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MATE	multidrug and toxic efflux
MDR	Multidrug resistance
MF	Major facilitator
MIC	Minimum inhibition concentration
mRNA	Messenger ribonucleic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
MS/MS	Tandem mass spectrometry
NACL	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
pH	Power of hydrogen
PRP's	Pentapeptide repeat proteins
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
SM	Saline-magnesium
SMR	Small multidrug resistance
spp	Species
TCEP	Tris(2-carboxyethyl) phosphine

TFA	Trifluoro acetic acid
TIC	Total iron chromatogram
TMD	Transmembrane domain
WHO	World Health Organization
SDGs	Sustainable Development Goals
ICs	Interfacial Contacts
SEDS	shape, elongation, division and sporulation

CHAPTER 1: INTRODUCTION

1.1. Background Information

The burden of communicable diseases potentiated by pathogenic bacteria remains a global concern as it contributes significantly to mortality (Ikuta *et al*., 2022). From community-acquired to nosocomial infection, pathogenic bacteria threaten the health and well-being of vulnerable groups including neonates, young children and patients that are immune-compromised, hospitalized or recovering from surgery (Folgori *et al.*, 2017). For instance, bacterial pneumonia and sepsis are the leading causes of mortality in children under the age of five, with 30% of sepsis cases among neonates ending in mortality (Folgori *et al.*, 2017). In 2017, there were 10 million deaths related to sepsis, representing 20% of the total global mortality rate (Rudd *et al*., 2020). In addition, antimicrobial resistance (AMR) has become a serious threat to public health and the medical industry. In 2014, the World Health Organization (WHO) in its Review on Antimicrobial Resistance, projected that by the year 2050 AMR could cause 10 million deaths annually (O'Neill, 2016). In 2019, AMR was directly involved in 1.27 million deaths while a further 4.95 million deaths were related to infections with bacteria that were resistant to antimicrobial drugs (Murray *et al*., 2022). In 2021, Ikuta and colleagues estimated that a staggering 4.71 million deaths were directly related to AMR, while a further 1.14 million were related to infection with antimicrobial resistant bacteria (Ikuta *et al*., 2022). Significantly, recently published data suggested that 33 bacterial pathogens, including drug resistant and sensitive strains, were directly responsible for 7.7 million deaths globally, which accounted for 13.6% of all deaths and 56.2% of all deaths related to sepsis, in 2019 (Ikuta *et al*., 2022). Of the 33 bacterial pathogens, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Streptococcus pneumoniae*, and *Pseudomonas aeruginosa* accounted for 54.9% of global deaths (Ikuta *et al*., 2022).

In addition to the human cost, AMR seems to exhibit an overwhelmingly high economic cost on low and lower-middle income countries (McDonnell *et al*., 2024). For instance, AMR currently costs South Africa US $ 0.5 billion as the country spends a larger proportion of their total healthcare budget on hospitalized patients (McDonnell *et al*., 2024). A strong association between AMR and increased costs and prolonged

hospitalization has been recorded (Serra-Burriel *et al.*, 2020). Some of the costs associated with AMR include an increase in the demand for medical equipment and supplies, sanitization of wards and beds and creation of isolation zones to prevent the spread of such pathogens (Serra-Burriel *et al.*, 2020). Antimicrobial resistance currently costs global economies US $ 66 billion and is projected to rise to US $ 159 billion if AMR rates conform to current trends (McDonnell *et al*., 2024). These statistics position AMR as a public health emergency that requires urgent intervention if we are to prevent the loss of human life and improve the economic burden that AMR presents.

The main contributors to development of AMR include the misuse or overuse of antimicrobials, limited availability of clean water and sanitation, and poor hygiene (WHO, 2023). Furthermore, antibiotics have been used extensively in agriculture since the early 1950s to prevent infection in livestock and crops produced for human consumption (Landers *et al.*, 2012). It has now been well documented that humans can acquire AMR through direct interaction with livestock and the environment, suggesting that the overuse of antimicrobials to improve health of livestock has also contributed to the development of AMR (Ma *et al*., 2021).

In addition, the limited development of new and efficient antibiotics is contributing to the surge in AMR (Nelson, 2003). The World Health Organization has stated that although 77 potential antibacterial drugs are in clinical development, the vast majority of these will not be approved (WHO, 2023). Antibiotic development depends heavily on expensive chemical methods to produce drugs that are rarely used as chronic treatment. Treatment lasts one to two weeks or at most a month, making antibiotics a less profitable investment when compared to medications developed for chronic ailments (Nelson, 2003). This is further complicated by the nature of AMR and the ability of bacteria to develop resistance to drugs. The World Health Organization has therefore revised the Bacterial Priority Pathogens List (BPPL) for 2024 to direct the development of new drugs aimed at combating the bacteria that currently pose the greatest threat to public health (WHO, 2024). In the BPPL bacterial pathogens are classified into three priority groups: critical, high and medium. The WHO defines the critical group as “Bacterial pathogens that pose the highest threat to public health due to limited treatment options, high disease burden and increasing trends in antibacterial resistance (ABR), with few or no promising candidates in the pipeline” (WHO, 2024).

This group consists of *Acinetobacter baumanni* and Enterobacterales such as *K. pneumoniae*, *E. coli* and *Proteus* species. The high group is defined by the WHO as “Bacterial pathogens that are significantly difficult to treat, cause a substantial disease burden, show increasing trends in resistance, are uniquely difficult to prevent, are highly transmissible and for which there are few potential treatments in the development pipeline” (WHO, 2024). This group consists of organisms including, but not limited to, *S. aureus* and *Enterococcus faecium*. While the development of antibiotics is an essential component of the modern healthcare system, the current pipeline for new, efficacious drugs to treat bacterial infections is insufficient (WHO, 2023). Consequently, there is an urgent need for targeted interventions and effective treatment strategies to combat these pathogenic bacteria.

Recently, there has been a significant increase in interest in bacteriophages due to their ability to induce the lysis of bacterial cells. Bacteriophages are viruses that specifically target and kill bacteria without any undesirable effects on human cells (Principi *et al*., 2019). Bacteriophages have co-evolved with bacteria for millions of years, developing a dynamic interaction that has resulted in reciprocal evolution, driving ecological and metabolic processes in microbial communities (Koskella and Brockhurst, 2014). The mechanism by which bacteriophages infect bacteria differs between each family. Briefly, the bacteriophage using receptor binding proteins, recognizes receptors on the surface of bacteria to which they bind (Egido *et al*., 2022). This process triggers the release of genetic material into the bacterial host cytoplasm and through a cascade of events, the bacteriophage hijacks the hosts genes to induce the replication of its own genome. This results in the expression of lytic proteins including holin and endolysins that facilitate the lysis of the bacterial host (Egido *et al.,* 2022). The clinical significance of bacteriophages is rooted in the production of endolysins and holins. Endolysins are peptidoglycan hydrolyzing enzymes produced by the viral progeny while holins facilitate the action of the endolysin by allowing access to the peptidoglycan layer by creating holes or rafts within the cytoplasmic membrane (Loessner *et al.,* 2005). These proteins have been identified as potential antimicrobials to aid or even replace current antibiotics. Given the substantial threat that AMR poses to public health and achievement of the Sustainable Development Goals (SDGs), bacteriophages and their associated proteins offer an attractive area

of study to combat the burden this issue presents not only to public health but also to agriculture, food security and hospitality.

1.2. Aim and objectives of the study

The primary aim of the present study was to evaluate the bacteriolytic and bactericidal activity of bacteriophage proteins against *Enterobacteriaceae* and *Staphylococcus aureus.*

The objectives were as follows:

1.2.1. To isolate bacteriophage associated proteins (lysins included) from *Escherichia coli* infected by bacteriophage T4.

1.2.2. To evaluate the antimicrobial activity of the isolated proteins against a host of critical and high priority bacterial pathogens including *Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli, Proteus mirabilis* and *Enterococcus faecalis.*

1.2.3. To encapsulate isolated proteins within a liposome and evaluate their ability to impede the growth of *Enterobacteriaceae* of critical and high priority.

1.2.4. To compare the antimicrobial activity of encapsulated lysins against purified lysins to determine if encapsulation enhances antimicrobial activity.

1.2.5. To use computational modeling to investigate the underlying molecular interactions between the endolysin and the penicillin binding proteins from *Staphylococcus aureus*.

1.3. Outline of the dissertation

The isolation and assessment of the antimicrobial activity of bacteriolytic and bactericidal endolysins of *Enterobacteriaceae* infecting bacteriophages.

Chapter one provides the background to the study as well as the aims and objectives. The impact and consequences of antimicrobial resistance are discussed. This chapter highlights some of the causes of antimicrobial resistance and introduces bacteriophages and their proteins as a possible alternative to traditional antibiotics.

Chapter two provides a literature review on the origins of antimicrobial resistance and discusses methods used by bacteria to evade the action of antibiotics. This chapter also provides a detailed explanation on bacteriophages, their proteins, in particular the structure and classification of the endolysin protein. Recent advances and research outcomes from published articles surrounding bacteriophage enzymes are also discussed.

Chapter three focuses on the methods used to isolate the bacteriophage T4 proteins. Detailed steps are provided. This chapter also focuses on the methods used in the proteomic analysis of the isolated proteins. The findings from the proteomic analysis are also examined and discussed.

Chapter four discusses the methods used in the formation of bacteriophage T4 loaded liposomes as well as the method used to determine the antimicrobial activity of the T4 loaded liposome and the T4 protein. The results of these experiments are presented and discussed. The prediction of molecular Interactions using molecular modeling between the T4 protein and active sites of the peptidoglycan layer is also discussed.

Chapter five provides a conclusion to the study. The impact of antimicrobial resistance and the findings of this study are briefly revisited. This chapter also provides suggestions for future investigation.

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction

Antimicrobial drugs are those that are deployed with the primary aim of reducing the level of infection and sepsis. Antimicrobial resistance is a natural process caused by complex changes in genetic material and consists of mechanisms that serve to protect the bacterial cell from destruction (Munita and Arias, 2016). Resistance is achieved by a bacterial population when the concentration of an antibiotic needed exceeds the amount that the infected host can tolerate (Schuffler *et al.*, 1994). Antimicrobial agents exert their activity through various pathways, from which they are categorized. For instance, they may disrupt cell wall formation through inhibition of transpeptidation, or they may inhibit protein and nucleic acid synthesis (Reygaert, 2018). Bacteria have developed methods of evading these modes of actions rendering such antimicrobial agents ineffective at therapeutic concentrations. These resistance mechanisms may be naturally present (intrinsic) or may be acquired through genetic mutation or the integration of exogenous genes from the environment into the bacterial genome (Hawkey, 1998).

2.1.1. Natural resistance

Certain bacteria are capable of proliferation despite the addition of antibiotics to the system (Reygaert, 2018). Such organisms are intrinsically resistant to antimicrobial agents. Gram-negative bacteria exhibit a peptidoglycan layer encapsulated by a lipopolysaccharide layer. The lipopolysaccharide layer is impermeable to large glycopeptide molecules such as vancomycin and teicoplanin, this protects the glycopeptide cell wall from antibiotics (Coculescu, 2009). Another example of natural resistance are anaerobes, which lack a transport system for antibiotics and are therefore resistant to the actions of aminoglycosides (Coculescu, 2009). Certain organisms may be naturally resistant to multiple drug classes due to the presence of more than one intrinsic resistance mechanism. Such mechanisms can be a low permeable cell wall, synthesis of beta-lactamase and efflux pumps. For instance, *Pseudomonas aeruginosa* is naturally resistant to aminopenicillin, certain cephalosporins and kanamycin (Livermore, 2002).

2.1.2. Acquired resistance

Acquired resistance is achieved when bacterial DNA mutates, and the expression of a new phenotypic trait occurs (McManus, 1997). Acquired resistance may occur following the inheritance of foreign DNA through horizontal gene transfer, or an organism's own chromosomal DNA may mutate to confer bacterial resistance (Cloeckaert *et al.*, 2017). *Acinetobacter* species are naturally competent and can therefore readily acquire genomic material from its environment (Reygaert, 2018). Genetic mutation often occurs due to stress (Foster, 2005), which may be caused by starvation, ultraviolet radiation, and/or chemicals. Mutations conferring resistance commonly occur in genes that code for traits that antibiotics rely on, such as genes encoding drug targets, drug transporters and their regulators, and those genes encoding antibiotic modifying enzymes (Martinez, 2014).

2.2. Mechanisms of resistance

Gram-positive and Gram-negative bacteria exhibit differing structural features that (Figure 2.1) create variation in the way these organisms resist the action of chemical destruction. The four main mechanisms used by bacteria to evade the action of antimicrobials include reducing drug uptake, altering drug targets, inactivating drugs, enhancing drug efflux (Chancey *et al.*, 2012; Martinez, 2014). The lack of a liposaccharide layer in Gram-positive bacteria limits its resistance capability. Gram-positive bacteria are rarely found to limit the uptake of a drug (Reygaert, 2018).

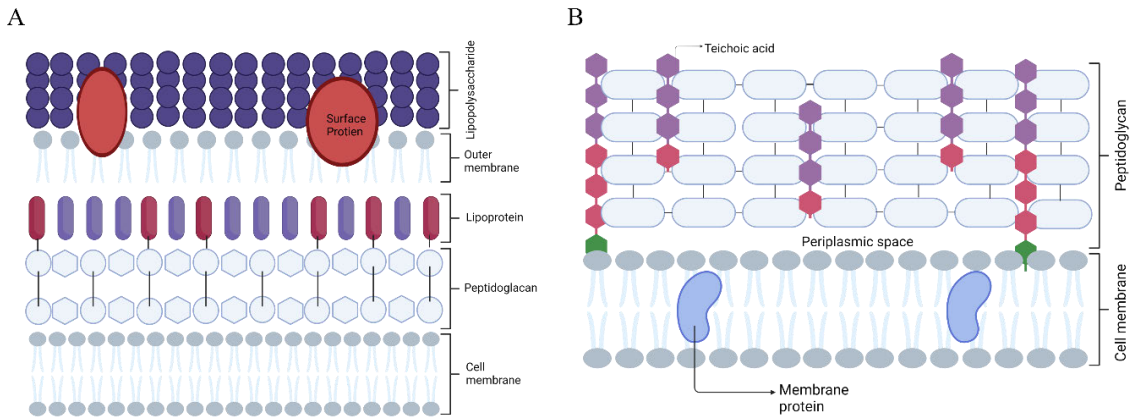

Figure 2.1: Differences in Gram-negative (A) and Gram-positive (B) bacterial cell walls (Adapted from Huan *et al.*, 2020).

2.2.1. Efflux pumps

A decrease in intracellular concentration of antibiotics can be achieved by intrinsic bacterial resistance mechanisms such as the development of a low permeability external layer which diminishes entry of the antibiotic (Nikaido, 2003). A bacterial cell can also attain this low intracellular antibiotic concentration by means of efflux pumps. Efflux pumps represent a family of transporter proteins localized in the cytoplasmic membrane and can transport a single or range of antibiotic substrates (Li and Nikaido, 2004). These pumps are associated with multiple drug resistance due to their ability to pump out antibacterial and toxic agents from within the cell into the extracellular environment thereby decreasing intracellular concentrations of antimicrobial agents (Webber, 2003).

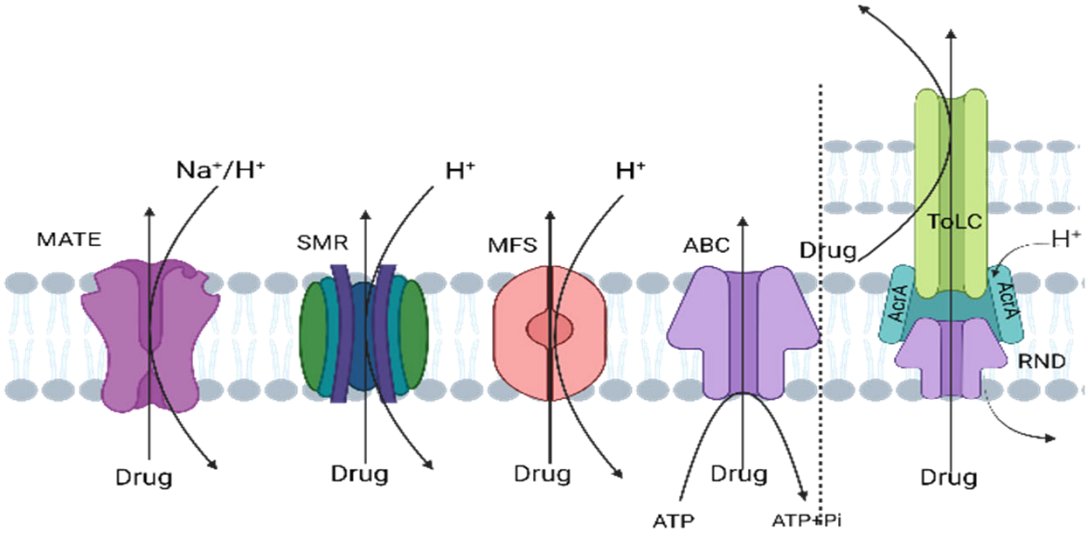

Figure 2.2: The five major efflux pumps in bacteria (Adapted from Alav *et al.,* 2018).

The five major families of efflux pumps include the major facilitator (MF), multidrug and toxic efflux (MATE), resistance-nodulation-division (RND), small multidrug resistance (SMR) and ATP binding cassette (ABC) (Figure 2.2) (Lomovskaya *et al.*, 2001). Major facilitator pumps display activity over a narrow range of substrates and are present in both Gram-positive and Gram-negative bacteria. Resistance-nodulation-division pumps are exclusively found in Gram-negative bacteria and display poly-selective, activity against several antibiotic classes, antiseptics, and detergents (Mahamoud *et al.*, 2007). The RND pumps in some Gram-negative organisms such as AcrB in

Escherichia coli and MexB in *Pseudomonas aeruginosa,* and their association with other protein groups such as the outer membrane channels, TolC and OprM and periplasmic proteins AcrA and MexA in *E. coli* and *P. aeruginosa*, respectively, play essential roles in resistance mechanisms (Paulsen *et al.*, 1997). Efflux pumps together with their characteristic lipophilic outer membrane in Gram-negative bacteria display a synergistic effort in protecting the bacterial cell from chemical destruction (Nikaido, 1996). The design and location of the three protein associations suggest that toxins are expelled into the extracellular space, rather than the periplasm. This means that the drug must pass the outer membrane once again (Nikaido, 1996). The intrinsic resistance to lipophilic drugs such as penicillin G, for instance, observed in Gram-negative bacteria was long thought to be a result of exclusion by the outer membrane (Vaara, 1993). However, in a study where the RND pump of *Salmonella typhimurium* was genetically inactivated, the MIC of lipophilic penicillin and cloxacillin for the organism was reduced from 512 µg/ml to 2 µg/ml. This suggested that the efflux pumps contributed greatly to multidrug resistance (Nikaido, 1996).

2.2.2. Biofilms

Biofilms are a multicellular community of bacterial cells that are confined within a matrix. This phenomenon commonly occurs in aqueous environments (Lopez *et al.*, 2010). Colonizing bacteria adhere to inert or living tissues and produce an exopolysaccharide layer composed of cellulose, alginates, poly-N-acetylglucosamine (PNAG), extracellular teichoic acid and several proteins and lipids (Jolivet-Gougeon and Bonnaure, 2014). The high yield of bacterial cells protected by the biofilm facilitates many pathways for the development of drug-resistant bacteria. In such densely populated colonies of bacterial cells, the number of resistant cells increases, and so does the transfer of genetic material (Lazar and Chifiriuc, 2010). Biofilms also provide a short-term protective layer preventing the entry of antimicrobials (Jolivet-Gougeon and Bonnaure, 2014). Biofilm forming colonies on medical devices such as urethral catheters and prosthetic material tend to find niches where they are protected from the action of antibiotics (Jolivet-Gougeon and Bonnaure, 2014). Antibiotic degrading enzymes accumulate under the biofilm. For example, Bagge and colleagues observed beta-lactamase gene promoter activity via the expression of the green fluorescent protein after six days of exposure to 100 mg/ml (Bagge *et al.*, 2004). Furthermore, persister cells are a common occurrence in biofilms whereby cells enter

a dormant non-dividing and low metabolic state. Persister cells become highly resistant to the action of antibiotics due to their lowered metabolic rate (Lewis, 2012).

2.2.3. Drug inactivation methods

Antimicrobials can be rendered ineffective via enzymatic hydrolysis, group transfer and redox processes resulting in metabolic degradation of the drug (Davies, 1994). A classic example of drug inactivation employed by bacteria is the synthesis of β-lactamases. Penicillin, cephalosporins and monobactams form the β-lactam group of antibiotics and are one of the most important antibiotic groups used to treat bacterial infection, which before the discovery of penicillin could have been fatal to patients (Tahlan and Jensen, 2013). The cell wall plays an essential role in preventing osmotic rupture of the cytoplasmic membrane. Its structure is formed from peptidoglycan consisting of glycan chains with peptide ends called penicillin-binding proteins forming the crosslink to the adjoining glycan (Hongbaek *et al.*, 2014). β-lactam antibiotics disrupt cell wall synthesis by binding to the glycan peptide ends preventing cross-linking (Blair *et al.*, 2014). The discovery of β-lactamases was made prior to the introduction of penicillin for clinical use. In the late 1940's, strains of *S. aureus* were found to synthesise the enzyme (Abraham and Chain, 1940). Penicillin is rendered ineffective after the enzyme catalyses the hydrolysis of the β-lactam ring (Palzkill, 2012).

Antibiotics are also susceptible to inactivation by altering their structure via the transfer of functional groups (Wright, 2005). The structural modification is catalysed by an enzyme and is permanent, preventing the antibiotic from binding to its target. Several groups of antibiotics including the aminoglycosides and macrolides are among the antibiotics susceptible to this resistance mechanism (Davies, 1994).

The third mechanism of inactivation of an antibiotic is by redox processes where a single hydroxyl group is transferred to tetracycline at position 11a. The transfer is facilitated by a flavin-dependent mono-oxygenase enzyme TetX. Tetracyclines ability to sequester Mg+ ions are critical for binding to its target and is hindered by this structural change (Park and Levy, 1988).

2.2.4. Changes in antimicrobial targets by modification and mutation

Some bacteria have developed the ability to make changes to the target sites while maintaining its normal function. This mechanism prevents the efficient binding of antibiotics conferring resistance (Munita and Arias, 2016). Resistance to antibiotics conferred by a single point mutation in pathogenic strains of bacteria can be clinically alarming because of the bacterial diversity found during an infection. Strains carrying the mutation can proliferate rapidly because the genes that encode antibiotic targets for some bacteria exist in multiple copies. The ribosomal subunit 23S rRNA in Gram-positive bacteria is the target for linezolid and is encoded by multiple identical copies of its gene (Blair *et al.*, 2014). *Streptococcus pneumoniae* and *S. aureus* have developed a mutation conferring resistance to linezolid in one of these copies and can transfer the chromosome during conjugation, rapidly producing a population with the resistance mechanism (Billal *et al.*, 2011). Another example of target modification is the methicillin-resistance observed in *S. aureus* (MRSA). Unlike penicillin resistance, resistance to methicillin does not involve β-lactamase or any alteration to native penicillin-binding sites. The resistance mechanism is due to the attainment of a second penicillin-binding protein referred to as PBP2a (Peacock and Paterson, 2015). This protein facilitates the construction of the peptidoglycan cell wall despite the ability of the antibiotic to inhibit the native PBP. This is due to the reduced affinity of PBP2a to methicillin (Peacock and Paterson, 2015).

DNA gyrase and topoisomerase IV are the enzyme targets of quinolone antibacterial agents. DNA gyrase is responsible for DNA supercoiling and the relief of topological stress created during the translocation, transcription, and replication complexes along DNA. Topoisomerase IV resolves interlinked daughter chromosomes following DNA replication (Drlica and Zhao, 1997). DNA gyrase and topoisomerase IV are both crucial for cell growth and replication. The *qnr* resistance gene present in various pathogens encodes pentapeptide repeat proteins (PRP's). The binding action of PRP's to DNA gyrase and topoisomerase IV provides protection from the action of quinolone antibacterial agents (Blair *et al.*, 2014). A recent model of the mechanism of action of PRP's suggests that the interaction of the PRP's with topoisomerase IV occurs after drug binding to stimulate the release of the quinolone (Vetting *et al.*, 2011). The release of the quinolone drug allows topoisomerase IV to carry out its intended function.

Colistin, also known as polymyxin B and polymyxin E, was one of the first antibiotics to demonstrate substantial and rapid bactericidal activity against Gram-negative bacteria, importantly *Pseudomonas aeruginosa* (Roger *et al.*, 2009). Despite its potent activity, its use in the 1970's was hindered mainly due to its nephrotoxic and neurotoxic effects, and it was replaced by aminoglycosides (Falagas *et al.*, 2005). In recent years a lack of alternate therapy owing to multidrug resistance has led to the widespread use of cyclic antimicrobial peptides in the treatment of infection (Blair *et al.*, 2014). Although the use of colistin has been relatively low, cases of infection with resistant strains of bacteria have been identified (Antoniadou *et al.,* 2007). Antimicrobial action of colistin is based on the binding of the hydrophobic chain to, and the disruption of, the lipopolysaccharide layer. Resistance is conferred when the expression of lipopolysaccharide production regulators is genetically altered resulting in overexpression of *pmrC* which alters lipid A (Adams *et al.*, 2009). The mutations result in the addition of phosphoethanolamine to lipid A, lowering the negative charge of the lipopolysaccharide layer. Colistin binding is reduced by a lowered negative charge (Beciero *et al.*, 2011).

2.3. Bacteriophages

The discovery of bacterial resistance was reported shortly after the approval of antibiotics for clinical use (Medina and Pieper, 2016). Seven hundred thousand deaths due to infection with resistant bacteria are recorded each year, with this number expected to reach 50 million by 2050 if no new antibiotics are discovered (Gabrowski *et al.*, 2021). In recent years the scientific community has gained an increasing interest in bacteriophages, especially with pan drug resistance on the rise (Lu *et al.*, 2020). The study of lytic bacteriophages may help develop novel antibiotics to treat these bacterial infections. Despite the multiple possible applications of bacteriophages in human medicine, the limited host specificity and the obvious development of phage resistance has limited its use in modern medicine (Zdorovenko *et al.*, 2018). In contrast, lytic enzymes encoded by bacteriophages may provide an attractive source of novel antimicrobials. Lytic enzymes display activity against a wider range of substrates and their use should not lead to bacterial resistance (Borysowski *et al.*, 2006). The two most vital proteins involved in host lysis by bacteriophages are, as mentioned earlier, endolysins and holins.

2.3.1. Holins

Holins are small hydrophobic proteins up to 130 amino acids in length. Holins accumulate as inactive homodimers in the inner membrane of infected bacterial cells and function to form holes in the cell membrane to allow endolysins access to the periplasm. Holins begin to aggregate to form rafts once critical concentrations are reached (Young, 1992). Depolarization occurs in the membrane due to the raft's permeability to ion flow resulting in the formation of holes by the aggregated holins (Savva *et al.*, 2014). Holins are divided into three classes according to the number of hydrophobic transmembrane domains (TMD's) they possess (Gabrowski *et al.*, 2021). Class I holins range between 95 and 135 amino acids in length and possess three TMD's; class II range between 65 and 95 amino acids in length and possess two TMD's; class III holins have only one TMD (Gabrowski *et al.*, 2021).

2.3.2. Endolysins

Endolysins are bacterial peptidoglycan hydrolyzing enzymes synthesized by dsDNA bacteriophages. The enzyme degrades bacterial peptidoglycan for release of viral progeny into the environment (Nelson *et al.*, 2012). The peptidoglycan layer of bacteria is composed of alternating *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) residues that make up a glycan polymer (Nelson *et al.*, 2012). An amide bond between MurNAc and a peptide secures the peptide to the glycan layer (Nelson *et al.*, 2012). Endolysins can be classified according to the peptidoglycan component which they cleave (Figure 2.3). For instance, *N*-acetylmuramidases, endo-β-*N*-acetylglucosaminidases and lytic transglycosylates are responsible for cleavage of a peptidoglycan sugar moiety. Endopeptidases on the other hand, cleave the peptide moiety while *N*-acetylmurmoyl-L-alanine amidases cleave the amide bond between the two moieties (Young *et al.,* 2000; Nelson *et al.*, 2012).

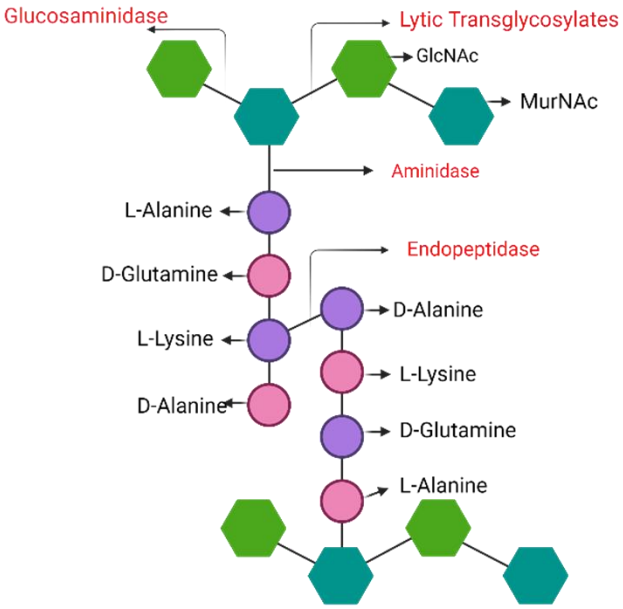

Figure 2.3: The bacteriophage enzymes and the peptidoglycan components which they degrade (São-José, 2018).

2.4. Recent advances in bacteriophage research

Given the renewed interest in bacteriophage therapy, there have been several publications highlighting their effectiveness as a potential antimicrobial agent (Table 2.1). Owing to advances in recombinant DNA, recombinant bacteriophage enzymes have been created and assayed against a host of drug resistant and sensitive pathogens (Table 2.1). Endolysin and holin have been cloned and characterized from the *Streptomyces avermitilis* bacteriophage phiSAD1 (Lu *et al*, 2020). The *S. avermitilis* phage phiSASD1 was isolated, sequenced and the endolysin expressed and isolated. Endolysin displayed strong lytic activity with up to 3 log reduction of viable cells against *S. avermitilis* as well as *Sarcina lutea* and *Bacillus subtilis* (Lu *et al.*, 2020). Moderate lytic activity, up to 1.8 log reduction was displayed against *E. faecalis* and *S. aureus.* However, the recombinant enzyme showed no antimicrobial activity against the Gram-negative bacteria (Lu *et al.*, 2020). Given the importance of cephalosporins and carbapenems as the final treatment options against pathogenic bacteria, the development of resistance to these drugs poses a serious threat to public health. (Moolchandani *et al.*, 2017). Since the lipopolysaccharide outer membrane in Gram-negative bacteria makes them less susceptible to endolysin proteins than Gram-

positive bacteria an outer membrane permeabilizer such as ethylene diamine tetra acetic acid (EDTA) is required for the endolysin protein to access the peptidoglycan layer in these bacteria (Oliviera *et al.*, 2014). A novel endolysin from a *Salmonella* spp. lytic bacteriophage, phage SS3e was cloned, expressed and the recombinant protein purified and analysed for its antimicrobial activity. The endolysins antimicrobial activity was examined against a host of multidrug resistant (MDR) Gram-negative bacteria (Kim *et al*., 2020). This study demonstrated bactericidal activity without the use of an outer membrane permeabilizer against MDR *Acinetobacter baumannii,* MDR *Escherichia coli,* MDR *K. pneumoniae* and MDR *Pseudomonas aeruginosa* (Kim *et al.*, 2020).

Table 2.1: Selected Endolysins with antibacterial activity against clinical bacteria discovered to date.

Endolysin	Bacteriophage	Family	Host	Active against	Reference
LysPA26	JD010	*Podoviridae*	*Pseudomonas aeruginosa*	*P. aeruginosa A. baumannii, K. pneumoniae*	(Guo *et al*., 2017)
LysH5	vB_SauS-philPLA88		*Staphylococcus aureus*	*S. aureus, S. epidermidis,* Anti-biofilm activity	(Gutierrez *et al*., 2014)
LysPBC1	PBC1	*Siphoviridae*	*Bacillus cereus*	*B. cereus*	(Kong and Ryu, 2015)
LysSP1	SLMP1	*Siphoviriae*	*Salmonella typhimurium*	*S. typhimurium, S. enteritidis, S. dysenteriae, E. coli, E. coli* O157	(Jiang *et al*., 2021)
LysJH307			*Streptococcus bovis*	*S. bovis* JB1, *S. equinus*, *S. alactolyticus*, *E. faecalis*, *B. subtilis*	(Kim *et al*., 2020)

Table 2.2: Endolysins and bacteriophages effective against food borne pathogens.

Food type	Endolysin	Bacteriophage	Activity	Reference
Dairy	DLn1	DLn1	Reduces the amount of *B. cereus* in milk over 6 h	(Li *et al*., 2022)
	LysH5	ΦH5	Reduced 106 CFU/ml of *S. aureus* to undetectable in 4 h	(Obeso *et al.,* 2008)
Dairy and raw beef		JN01	Significantly reduced *E. coli* O157:H7 in milk and raw beef	(Li *et al.,* 2021)
Meat/poultry	LysSE24	LPSE1 *Salmonella* phage	LysSE24 showed lytic activity against *Salmonella enteriditis* as well as 23 other multidrug resistant *Salmonella* spp.	(Ding *et al.,* 2020)
Meat/poultry and vegetables		SalmoFresh™ bacteriophage mix from Intralytix	SalmoFresh™ is a blend of six lytic bacteriophages, active against *Salmonella enterica*	(SalmoFresh™)

Some organisms implicated in food contamination are *E. coli, Salmonella* species, *Clostridium* species and *Listeria monocytogenes.* Contamination of food can occur at any time during the processing from raw agricultural products to distribution, markets, purchase and use by consumers. The introduction of bacteriophage enzymes at different points can help reduce the effects of bacterial contamination. There have been several studies outlining the efficacy of endolysins on bacteria implicated in food born illnesses (Table 2.2). Bovine mastitis is one of the most challenging diseases in dairy production where up to 75% of all bovine mastitis cases are caused by *Staphylococcal* and *Streptococcal* species. A chimeric endolysin, λSA2-E-Lyso-113 SH3b and λSA2-E-LysK-SH3b consisting of an endopeptidase domain of a *Streptococcal* phage endolysin λSA2 and the cell binding domain from a *Staphylococcal* bacteriophage endolysin LysK had bactericidal activity. The chimeric endolysins demonstrated antibacterial activity against several mastitis causing *S. aureus* strains when 1 µg protein was applied to bacterial lawns. Visible lysis zones were demonstrated (Smelcher *et al.*, 2012). Furthermore, the endolysin displayed bactericidal activity in cow's milk, after 100 µg enzyme was added to cow's milk simultaneously inoculated with 2 x 10^3 and 2 x 10^6 CFU/ml *S. aureus*, where the endolysin reduced bacterial numbers over the course of 3 h (Smelcher *et al.*, 2012).

2.5. Application in agriculture

Antibiotics have been used to reduce infection in animal and crop production as well as maintain the health of livestock in confined spaces. This use of antibiotics within the agricultural sector has led to the spread of multidrug resistant strains of pathogens. This risks transmission of these pathogens to humans through consumption or direct contact through handling and preparation of contaminated food products (Chang *et al.*, 2015). The use of animal faecal waste as a method of increasing soil fertility has facilitated the transfer of antibiotics into the environment, providing the conditions necessary for bacteria to develop additional antimicrobial resistant genes (Manyi-Loh *et al.,* 2018).

Studies highlighting the efficacy of endolysins against plant pathogens are limited. There was significant reduction in *Ralstonia solanacearum* cells when treated with three lytic bacteriophages, RSA1, RSB1 and RSL1 (Fujiwara *et al.*, 2011). *Ralstonia solanacearum* is a Gram-negative bacterium found in soil, causing bacterial wilt in

plants (Genin and Denny, 2012). This bacterium has a wide host range including economically important crops, such as tobacco and tomato (Fujiwara *et al.*, 2011).

2.6. Endolysin as a potential antimicrobial agent

As the occurrence of antimicrobial resistance grows, bacteriophage endolysins have been suggested as an alternative antimicrobial, owing to several advantageous properties (Ajuebor *et al.*, 2018). Among these properties are the enzymes narrow host specificity, allowing commensal flora to continue thriving (Smelcher *et al*., 2012). The coevolution of bacteriophages and bacteria has allowed endolysins to develop the ability to cleave highly conserved components of the peptidoglycan layer (Zdorovenko *et al.,* 2018). Resistance mechanisms such as active efflux pumps and reduced membrane permeability are avoided by the application of endolysin as an antimicrobial since the enzyme can hydrolyze the peptidoglycan externally (Viertal *et al.*, 2014).

Despite the advantages of endolysins, there are still limitations and challenges to its practical use. The development of endolysins requires several time-consuming and costly steps, including bacteriophage isolation, propagation, and endolysin purification (Viertal *et al.*, 2014). While these may be relatively easy in a laboratory setting, industrial settings may find it challenging. Given that the main aim of propagation of bacteriophages for endolysin is to eradicate pathogenic bacteria, having to cultivate such strains on a large scale to cater for the specificity of bacteriophages creates the potential for spread of infection and carries a high financial burden on pharmaceutical companies (Son *et al.*, 2021). Although the topical application of endolysin for the treatment of skin infections is a common practice, the protein nature of the endolysin will quickly be denatured by enzymes, differing pH levels and mechanical digestion when administered orally to target internal infections (Son *et al.*, 2021). Intravenous (IV) administration of endolysins may overcome the challenges posed by oral administration, however, this method is undesirable due to its invasiveness and inconvenience to patients not hospitalized who will need a specialized clinic for administration (Son *et al.*, 2021).

As researchers and scientists look to the future of endolysins as antimicrobials it is important to find ways to overcome their limitations, perhaps the advancements in biotechnology and enzymology may be able to aid in these matters. The isolation of

genes encoding endolysins for the creation of plasmids to transform non-pathogenic host bacteria to impart the ability to produce endolysin will eliminate the need to cultivate pathogenic bacteria. Encapsulation of enzymes within nanoparticles, such as solid lipid nanoparticles, may improve the enzymes stability and ability to withstand oral administration.

In this study, the endolysin from the *E. coli* T4 bacteriophage was isolated and encapsulated within a liposome to determine the effects of encapsulation on the efficacy of the protein's antimicrobial properties.

CHAPTER 3: COMPLETE PROTEOME OF BACTRIOPHAGE T4 AND IDENTIFICATION OF ACTIVE FRACTIONS

3.1. Background Information

Antimicrobial resistance has been recognized as a global health emergency that necessitates immediate intervention through the development of new drugs aimed at minimizing mortality and mitigating the impact of infectious diseases on achievement of the SDGs. In light of this fact, bacteriophages and their proteins, given their ability to interact with bacteria bringing about their lysis, may have a significant role to play in delivering much needed therapeutic relief from AMR. A thorough understanding of the proteome of bacteriophages can reveal potential targets for new drugs and assist in characterizing phage-derived proteins. As such, this chapter focused on elucidating the total phage proteome of *Escherichia* bacteriophage T4 by mass spectrometry in order to facilitate the identification of protein biologics. Detection of the endolysin, with the ability to disrupt bacterial cell walls from proteomic analysis guided the separation of active fractions that were prepared by gel filtration targeted towards isolation of the endolysin.

3.2. Methodology

3.2.1. Study setting and design

Unless otherwise stated, the current study utilized an experimental study design. All research works were performed at the Research Centre for Plant Growth and Development. The research was approved by the Institutional Research Ethics Committee at The Durban University of Technology and was provided the Ethics Clearance Number: IREC 205/22.

3.2.2. Cultivation of bacteria and bacteriophage T4

Escherichia T4 bacteriophage (DSM 4505) along with its host, *E. coli* (DSM 613, ATCC 11303; Leibniz Institute DSMZ) formed the basis of the work. After reconstitution in nutrient broth, *E. coli* was cultivated on nutrient agar (1.5%; 5 g/l peptone, 3.0 g/l meat extract, 15 g/l agar (Sigma-Aldrich, Germany) and incubated for 24 h at 37°C. The next day, 10 ml of nutrient broth was inoculated with a single colony of *E. coli* and

incubated for 24 h at 37°C. One-hundred microliters of this culture were then transferred to nutrient agar (0.75%) to which the bacteriophage T4 (reconstituted in nutrient broth) was added and mixed gently. The mixture was then poured over solidified nutrient agar in 90 mm sterile plastic petri dishes and incubated at 37°C for 12 h. Following incubation, bacteriophage plaques were visible. The plaques were picked up and stored in saline-magnesium (SM) buffer (0.1 M NaCl, 8 mM $MgSO_4$, 50 mM Tris, pH 7.5) at 4°C up to two weeks.

3.2.3. Extraction of bacteriophage T4 proteins

Nutrient broth (100 ml) was inoculated with a colony of *E. coli* and incubated at 37°C overnight. The next day the broth was added to 1L of fresh nutrient broth and incubated for 1 h. Following the incubation period, 45 ml of SM buffer containing a high concentration of the bacteriophage T4 was added to the broth and the solution was mixed gently by inversion. The solution was then incubated at 37°C for 20 min. Post incubation, the broth was placed directly onto ice and then divided into 100 ml centrifuge tubes and centrifuged at 10 000 rpm and 4°C for 15 min. The supernatant was discarded and 6 ml of phosphate buffer together with 120 µl of Triton X-100 (Sigma-Aldrich, Germany) was added to the pellet and this was incubated at 37°C for 60 min. Six-hundred microliters of 0.005 M EDTA (Sigma-Aldrich, Germany) was added post incubation and centrifuged for 1 h at 10 000 rpm, the pellet was discarded and 300 µl of 0.3 M disodium-tetrathionate (Sigma-Aldrich, Germany) was added to the supernatant. The solution was placed in an ice bath and stirred constantly for 1 h. Subsequently, the mixture was saturated to 85% with ammonium sulphate (Sigma-Aldrich, Germany) and incubated at 4°C for 18 h. Following the 18 h incubation period the mixture was centrifuged at 10 000 rpm for 1 h and the protein sediment collected and resuspended in 5 ml of 0.05 M phosphate buffer (2.43g/l $NaH_2PO_4H_2O$, 5.65g/l Na_2HPO_4, pH, 6.1). The protein suspension was then dialysed through a cellulose dialysis membrane (Sigma-Aldrich, Germany) against 200 ml of phosphate buffered saline (16g/l NaCl, 0.40 g/l KCl, 0.49g/l KH_2PO_4, 5.36g/l Na_2HPO_4, pH, 6.0) overnight.

3.2.4. Proteomic analysis of active fractions

3.2.4.1. On-bead protein digestion

All chemicals and reagents used were of analytical grade. Protein samples were dissolved in Tris-HCl (100 mM) (Sigma-Aldrich) containing NaCl (100 mM) and sodium dodecyl-sulfate (1%) (Sigma-Aldrich, Germany). The solution was then reduced with 5 mM Tris-carboxyethyl phosphine (TCEP; Fluka) in 100 mM Tris-buffer for 1 h at 60°C. Cysteine residues were carbamidomethylated with 40 mM Iodoacetamide (Sigma-Aldrich, Germany) in 100 mM ammonium bicarbonate for 30 min at 25°C in the dark. After carbamidomethylating, 40 mM DTT was added to quench the reaction. The samples were subsequently diluted two-fold with a binding buffer made up of ammonium acetate (200 mM) and acetonitrile (30%) at pH 4.5. The protein solution was added to MagResyn (Resyn Biosciences) HILIC magnetic particles prepared according to manufacturer's instructions (equilibrated with 100 mM ammonium acetate, 15% acetonitrile, pH 4.5) and incubated for 30 min. After incubation, the supernatant was removed leaving behind magnetic particles. These were rinsed twice with acetonitrile (95%) and suspended to final ratio of 1:50 in 50 mM ammonium bicarbonate containing trypsin (Pierce). The solution was incubated for 18 h at 37°C. Peptides were extracted with 50 ml 15% trifluoro-acetic acid (Sigma-Aldrich). The samples were dried and re-suspended in 50 ml loading solvent for LC-MS/MS analysis.

3.2.4.2. Chromatographic conditions

Liquid chromatography was performed on a Thermo Scientific Ultimate 3000 RSLC. The instrument was equipped with a C18 trap column (5 mm x 300 mm) (Thermo Scientific). The following solvent systems were utilised: 2% acetonitrile: water; 0.1% formic acid (loading); solvent A consisted of water containing 0.1% formic acid and solvent B consisted of 100% acetonitrile with 0.1% formic acid. The samples were loaded onto the trap column using loading solvent (2 ml/min) at a constant temperature of 7°C. Loading was performed for 3 min, after which, the sample was eluted (300 nl/min) onto a CSH (25 cm x 75 mm x 1.7 mm) C18 analytical column (Waters). The gradient generated as follows: 5.0% - 30% solvent B over 60 min and 30 - 50% solvent B from 60 - 80 min. Chromatography was performed at 45°C. Mass spectrometry was

performed using a Thermo Scientific Fusion mass spectrometer equipped with a Nanospray Flex ionization source. The sample was introduced through a stainless-steel nano-bore emitter. Data was collected in positive mode with spray voltage set to 1.8 kV and ion transfer capillary set to 295°C. Spectra were internally calibrated using polysiloxane ions at m/z = 445.12003. MS1 scans were performed using the orbitrap detector set at 60 000 resolutions over the scan range 375-1500 with AGC target at 4 E5 and maximum injection time of 50 ms. Data was acquired in profile mode. MS2 acquisitions were performed using monoisotopic precursor selection for ions with charges +2 to +7 with error tolerance set to +/- 10 ppm. Precursor ions were excluded from fragmentation once for a period of 60 s. Precursor ions were selected for fragmentation in HCD mode using the quadrupole mass analyser with HCD energy set to 30%. Fragment ions were detected in the Orbitrap mass analyser set to 30 000 resolutions. The AGC target was set to 5E4 and the maximum injection time to 100 min. The data was acquired in centroid mode.

3.2.4.3. Database searching

All MS/MS samples were analyzed using MS-GF+ (University of California, San Diego, San Diego, CA, USA; version Release (v2024.03.26)) and X! Tandem (The GPM; version X! Tandem Vengeance (2015.12.15.2)). MS-GF+ and X! Tandem were directed to search the uniprotkb_2681598_concatenated_target_decoy database assuming the digestion enzyme trypsin. Fragment and parent ion mass tolerance measures were set to 10.0 PPM each for X! Tandem while for MS-GF+, a fragment and parent ion mass tolerance of 0.50 Da 10.0 PPM, respectively, were utilised.

3.2.4.4. Criteria for protein identification

Peptides and proteins were identified with Scaffold (version Scaffold_4.4.6, Proteome Software Inc., Portland, OR). Identities were considered valid only if they had a confidence score exceeding 95.0%. Peptide probabilities generated by MS-GF+ were allocated using the Scaffold Local FDR algorithm, while those from X! Tandem were assigned using the Peptide Prophet algorithm with Scaffold delta-mass correction (Keller *et al*., 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 4 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, 2003).

3.2.5. Separation of fractions and estimation of bactericidal activity via the spot assay

The protein suspension was separated via gel filtration chromatography on a Sephadex G-75 (Sigma-Aldrich, Germany) column with PBS yielding 50 fractions which were subjected to antimicrobial screening through the antibacterial spot-on assay (Fijan, 2016). All fractions derived from gel filtration were subjected to the spot assay to ascertain activity against *E. coli*. A colony of *E. coli* was cultivated overnight in nutrient broth at 37°C at 65 rpm. The next day, the culture was suspended in nutrient agar (0.75%) and poured over solidified nutrient agar (1.6%) and left to solidify. Bacteriophage T4 protein fractions were pipetted (10 µl) onto the top agar and left to dry. The plates were incubated at 37°C for 12 h. The formation of plaques was taken as bactericidal activity.

3.2.6. Estimation of total protein

The Bradford method was used to estimate total protein content of active fractions derived from gel filtration (Bradford, 1976). Briefly, 100 µl of protein extract was transferred into a clean test tube, to which 900 µl of PBS was added. One millilitre of the Bradford dye was added to the test tube and the solution was vortexed and allowed to stand for 5 min. The absorbance of 1 ml of the solution was read at 595 nm against a blank consisting of the PBS. Bovine serum albumin served as a control, from which a standard curve was generated to determine total soluble content of active fractions.

3.2.7. Data analysis

Unless otherwise specified, all experiments were conducted twice with three replicates each. Data pertaining to antimicrobial activity of bacteriophage and liposomal protein were subjected to a one-way analysis of variance. The lack of significant differences prevented the use of post hoc tests.

3.3. Results and Discussion

3.3.1. Proteomic features of *Escherichia* bacteriophage T4

As bacteriophages are being investigated as alternatives to antibiotics to counter the threat of AMR, a thorough understanding of their proteomic features can aid in the drug discovery process and elucidate the mechanisms by which the phage exerts its interaction. In the present study, the total phage proteome of *Escherichia* phage T4 post infection was investigated by mass spectrometry. A total ion chromatogram presented in Figure 3.1 provides evidence of successful digestion of the protein extract. Analysis was performed on two replicates, and a protein was only considered detected if it appeared in both protein profiles. As such, following the removal of duplicates and uncharacterized phage proteins, a total of 143 proteins were successfully detected by liquid chromatography (Table 3.1).

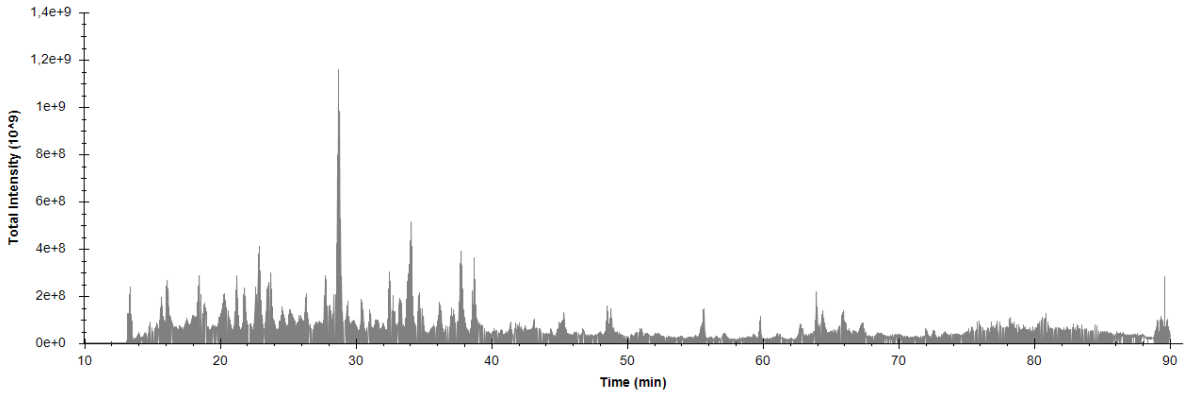

Figure 3.1: Total ion chromatogram (TIC) indicative of successful digestion of peptides.

The genome of bacteriophage T4 encodes approximately 300 gene products, however a significant number of these (approximately 130) remain uncharacterized (Zhang *et al*., 2021). Several structural proteins are ubiquitous throughout the T4 bacteriophages including an elongated head, tail and tail fibres that protrude from the baseplate (Black and Rao, 2012). The current study identified several significant structural proteins. Among them were three key gene products related to the capsid structure: gp20 (portal

vertex protein), gp23 (host specificity protein) and gp24 (vertex protein). These proteins play a key role in head assembly, whereby the association among 160 hexamers of gp23, 11 pentons of gp24 and one dodecamer of gp20 build the T=13 laevo capsid. In addition, gp20 binds to the *yidC insertase* on the cell membrane of *E. coli*, forming the prohead (gp21 protease) which is the first step in bacteriophage head assembly. The presence of gp21 (prohead core protein, Table 3.1) may be indicative of active and successful infection of *E. coli.* Other significant structural proteins that were identified include several that are involved in baseplate assembly. The baseplate is a crucial cluster of proteins involved in recognizing and binding to the host cell surface. For instance, the baseplate wedge protein (gp10) aids in unfolding the short tail fibres that interact with receptors on the host cell surface. These fibres bind irreversibly to the lipopolysaccharide's component of the cell surface once at least three long tail fibres have already attached.

Table 3.1: *Escherichia* bacteriophage T4 gene products detected via mass spectrometry.

Description of Gene Product	UNIPROT Accession Code	Coverage (%)	No. of Peptides	No of PSMs
AAA+ ATPase domain-containing protein	A0A7S9SWC3_BPT4	96.58	90	101
Activator of host PrrC lysyl-tRNA endonuclease	A0A7S9XHR2_BPT4	61.54	4	4
Activator of middle period transcription	A0A7S9SUD9_BPT4	83.41	57	64
Alpha glucosyl transferase	A0A7S9SVS8_BPT4	99.75	152	169
Anaerobic NTP reductase, large subunit	A0A7S9SVR6_BPT4	99.67	340	440
Anaerobic ribonucleoside-triphosphate reductase-activating protein	A0A7S9SVT6_BPT4	57.05	27	37
Anti-CBASS protein Acb1	A0A7S9SVH4_BPT4	47.37	8	8
Antiholin	A0A7S9SVV9_BPT4	95.88	55	67
Anti-restriction nuclease	A0A7S9SVN5_BPT4	87.76	64	75
Anti-sigma 70 protein	A0A7S9SVX9_BPT4	86.67	11	12
Baseplate hub	A0A7S9XHP8_BPT4	50.0	4	4
Baseplate hub assembly catalyst	A0A7S9SVK0_BPT4	86.75	45	50
Baseplate hub distal subunit	A0A7S9XH19_BPT4	76.27	23	27
Baseplate hub subunit	A0A7S9XHP4_BPT4	99.23	134	144
Baseplate hub subunit, tail length determinator	A0A7S9XEW9_BPT4	100.0	435	631
Baseplate tail tube cap	A0A7S9SVT9_BPT4	95.05	111	125
Baseplate tail tube initiator	A0A7S9SW23_BPT4	91.59	94	123
Baseplate wedge protein gp10	A0A7S9SU82_BPT4	86.88	180	213
Baseplate wedge protein gp6	A0A7S9SVW1_BPT4	94.7	305	377
Baseplate wedge protein gp7	A0A7S9SVH5_BPT4	97.77	769	1083
Baseplate wedge subunit	A0A7S9SVZ9_BPT4	81.14	67	76
Baseplate wedge subunit and tail pin	A0A7S9SW18_BPT4	47.95	8	8

Baseplate wedge tail fiber connector	A0A7S9SWG4_BPT4	89.58	38	41
Beta glucosyl transferase	A0A7S9SVJ0_BPT4	95.16	133	147
Capsid vertex protein (Gp24)	A0A7S9XH15_BPT4	83.61	65	71
Chaperone for tail fiber formation	A0A7S9SVZ1_BPT4	50.0	3	4
dCMP deaminase	A0A7S9SUB7_BPT4	79.27	26	27
dCMP hydroxymethylase	A0A7S9SVR4_BPT4	99.59	118	151
dCTPase	A0A7S9SVN9_BPT4	86.55	32	35
dihydrofolate reductase	A0A7S9XEY2_BPT4	48.7	11	13
Discriminator of mRNA degradation	A0A7S9XHZ5_BPT4	76.67	10	10
Distal long tail fiber assembly catalyst	A0A7S9XH32_BPT4	80.87	25	26
DNA end protector protein	A0A7S9SW09_BPT4	87.59	73	82
DNA endonuclease IV	A0A7S9SWL4_BPT4	73.51	21	23
DNA helicase	A0A7S9SV89_BPT4	94.08	116	136
DNA helicase assembly protein	A0A7S9SW56_BPT4	58.99	22	26
DNA ligase	A0A7S9SUA9_BPT4	93.02	208	227
DNA primase	A0A7S9SVS2_BPT4	94.15	126	144
DNA topoisomerase (ATP-hydrolyzing)	A0A7S9SVQ3_BPT4	71.25	21	22
DNA topoisomerase (ATP-hydrolyzing)	A0A7S9SVR7_BPT4	97.67	256	295
DnaB-like replicative helicase	A0A7S9SV99_BPT4	97.68	181	218
DNA-directed DNA polymerase	A0A7S9SVB6_BPT4	100.0	990	1420
DNMP kinase	A0A7S9SWF2_BPT4	84.65	36	40
DsDNA binding protein	A0A7S9SUD5_BPT4	44.94	7	8
DUF4326 domain-containing protein	A0A7S9XI43_BPT4	26.85	7	9
Endolysin	A0A7S9SVX7_BPT4	69.51	13	13
Endonuclease	A0A7S9SUF2_BPT4	64.06	4	4
Endonuclease V	A0A7S9SVQ9_BPT4	67.39	7	8
Exonuclease A	A0A7S9SVP3_BPT4	70.04	34	41
Frd2 protein	A0A7S9SW50_BPT4	83.59	35	44
Glutaredoxin	A0A7S9XES7_BPT4	39.22	3	3
Glycine radical domain-containing protein	A0A7S9XEU2_BPT4	65.83	9	9
Gp30.1	A0A7S9SW52_BPT4	95.51	13	13
Gp30.2	A0A7S9XGF0_BPT4	96.04	50	56
Gp30.3 protein	A0A7S9XI36_BPT4	74.34	14	17
Gp30.3' protein	A0A7S9SVK8_BPT4	17.33	2	2
Head assembly cochaperone with GroEL	A0A7S9SVL0_BPT4	25.23	7	8
Head completion nuclease	A0A7S9XGD3_BPT4	62.67	14	14
Head vertex assembly chaperone	A0A7S9XHK1_BPT4	49.12	5	6
Highly immunogenic outer capsid protein	A0A7S9SW08_BPT4	84.31	66	85
Hinge connector of long tail fiber, distal connector	A0A7S9SVN7_BPT4	96.38	31	32
Hinge connector of long tail fiber, proximal connector	A0A7S9XI46_BPT4	88.71	50	52
Holin	A0A7S9XEZ2_BPT4	87.16	36	39
Homing endonuclease	A0A7S9SVM3_BPT4	91.67	85	97
Host specificity protein	A0A7S9SVG2_BPT4	50.25	11	12
Immunity to superinfection membrane protein	A0A7S9SVL7_BPT4	92.77	10	12
Inhibitor of host transcription	A0A7S9SVW7_BPT4	68.86	16	17
Inhibitor of MrcBC restriction endonuclease	A0A7S9SVY7_BPT4	38.3	4	4
Inhibitor of prohead protease	A0A7S9SVU0_BPT4	72.12	20	20
Internal head protein	A0A7S9SVV0_BPT4	58.95	7	8

Late transcription coactivator	A0A7S9SWK3_BPT4	65.18	5	5
Long tail fiber, distal subunit	A0A7S9XHQ8_BPT4	99.9	703	961
Long tail fiber, proximal subunit	A0A7S9XGG4_BPT4	98.99	1055	1560
Lysis inhibition accessory protein	A0A7S9SVZ5_BPT4	59.76	7	7
Macro domain-containing protein	A0A7S9SU59_BPT4	95.48	30	31
Major capsid protein	A0A7S9SVJ4_BPT4	97.7	148	179
Modifier of transcription	A0A7S9XGX3_BPT4	45.06	6	6
Molybdenum ABC transporter	A0A7S9XGA5_BPT4	60.0	39	43
mRNA metabolism modulator	A0A7S9SV73_BPT4	88.65	25	26
NAD (+) --arginine ADP-ribosyltransferase	A0A7S9SW29_BPT4	96.33	321	398
NAD--protein ADP-ribosyltransferase modA	A0A7S9SU06_BPT4	82.0	20	22
NAD--protein ADP-ribosyltransferase modB	A0A7S9SVS4_BPT4	90.82	45	49
Neck protein	A0A7S9XI25_BPT4	93.2	68	74
Nuclease associated modular domain-containing protein	A0A7S9SUC5_BPT4	75.9	12	17
Nucleoid disruption protein	A0A7S9XH36_BPT4	98.01	26	26
Nucleotide reductase subunit C	A0A7S9XGC0_BPT4	95.28	24	24
Nudix hydrolase	A0A7S9SWE2_BPT4	78.81	43	52
OriE replication initiation protein	A0A7S9XH07_BPT4	92.0	10	14
Outer membrane lipoprotein Rz1	A0A7S9SVV5_BPT4	54.55	6	7
Outer membrane protein	A0A7S9XEZ5_BPT4	100.0	16	18
Packaging and recombination endonuclease VII	A0A7S9SVU7_BPT4	91.08	66	70
Phospholipase	A0A7S9SVS3_BPT4	49.48	4	4
Polynucleotide 5'-kinase and 3'-phosphatase	A0A7S9XH26_BPT4	86.71	59	69
Portal protein	A0A7S9SW10_BPT4	100.0	478	630
Pre-baseplate central spike protein Gp5	A0A7S9SVH6_BPT4	100.0	348	462
Prohead core protein (Gp21)	A0A7S9SWG8_BPT4	67.5	30	37
Prohead core scaffold protein	A0A7S9XI28_BPT4	75.84	27	27
Prohead core scaffold protein and protease	A0A7S9SW28_BPT4	80.66	28	33
Protease inhibitor	A0A7S9SU41_BPT4	81.37	16	16
Protector from prophage-induced early lysis	A0A7S9SVM1_BPT4	97.79	435	538
Protein gp30.7	A0A7S9SVV1_BPT4	76.86	21	24
Protein gp30.8	A0A7S9SW35_BPT4	41.82	11	13
Protein spackle	A0A7S9SVT7_BPT4	84.54	16	16
Putative anti-sigma factor	A0A7S9SW83_BPT4	55.24	40	46
Putative inner membrane	A0A7S9SW33_BPT4	83.33	46	60
Putative membrane protein	A0A7S9XGD1_BPT4	70.77	33	48
Putative periplasmic protein	A0A7S9SW85_BPT4	97.96	7	7
Putative regulatory protein FmdB Zinc ribbon domain-containing protein	A0A7S9XHY5_BPT4	65.52	8	9
RecA-like recombination protein	A0A7S9XGY1_BPT4	94.88	77	85
Recombination endonuclease subunit	A0A7S9SVA7_BPT4	97.14	320	394
Recombination, repair and ssDNA binding protein	A0A7S9SUA0_BPT4	62.77	21	25
Ribonuclease	A0A7S9SW77_BPT4	79.67	50	55
Ribonucleoside-diphosphate reductase	A0A7S9XGF9_BPT4	99.87	515	650
RIIA lysis inhibitor	A0A7S9SV84_BPT4	34.33	2	2
RNA ligase 1	A0A7S9SW04_BPT4	96.26	131	151
RNA ligase 2	A0A7S9XEW0_BPT4	73.95	59	73
RNA polymerase binding protein	A0A7S9XGB2_BPT4	84.5	35	46

RNA polymerase sigma-like factor	A0A7S9SU33_BPT4	96.76	54	60
RNA-DNA and DNA-DNA helicase	A0A7S9SVJ1_BPT4	97.81	273	358
Short tail fibres protein	A0A7S9XGD8_BPT4	96.2	210	257
Single-stranded DNA-binding protein	A0A7S9SW12_BPT4	97.67	139	172
site-specific DNA-methyltransferase (adenine-specific)	A0A7S9SVA6_BPT4	86.87	31	33
Site-specific RNA endonuclease	A0A7S9XI14_BPT4	67.97	15	19
Sliding clamp	A0A7S9SVU8_BPT4	91.23	47	53
Sliding-clamp-loader large subunit	A0A7S9SU28_BPT4	89.97	67	78
Sliding-clamp-loader small subunit	A0A7S9SWA0_BPT4	74.33	16	16
Small outer capsid protein	A0A7S9SVQ6_BPT4	75.0	6	6
Tail completion and sheath stabilizer protein	A0A7S9SU76_BPT4	65.91	10	10
Tail fibres protein	A0A7S9SW37_BPT4	41.18	6	6
Tail sheath protein	A0A7S9SVW9_BPT4	98.03	224	261
Tail sheath stabilizer and completion protein	A0A7S9XHN5_BPT4	93.01	56	63
Terminase DNA packaging enzyme, small subunit	A0A7S9XH11_BPT4	95.73	54	62
Terminase, large subunit	A0A7S9XEV4_BPT4	96.72	192	235
Thioredoxin	A0A7S9SVV3_BPT4	98.82	186	214
Thymidine kinase	A0A7S9SVT1_BPT4	79.27	25	28
Thymidylate synthase	A0A7S9XHQ5_BPT4	77.6	29	31
Topoisomerase II, medium subunit	A0A7S9XGG9_BPT4	95.25	116	133
Transcription modulator	A0A7S9XHJ7_BPT4	83.58	47	69
Transcription modulator under heat shock	A0A7S9XGX7_BPT4	93.17	29	32
Transcriptional regulator	A0A7S9SVG9_BPT4	92.17	25	26
Transglycosylase SLT domain-containing protein	A0A7S9XGC7_BPT4	70.17	27	30
Translation repressor protein	A0A7S9SVT4_BPT4	54.1	11	11
Valyl-tRNA synthetase modifier	A0A7S9SVY9_BPT4	66.96	11	11

In addition to structural proteins, and of particular interest to the current study, liquid chromatography was also able to detect the endolysin protein (Figure 3.2A). The endolysin is characterized by the ability to degrade the peptidoglycans present in the cellular membrane of the host. In association with the holin and its ability to permeabilize the cellular membrane, the endolysin reaches the periplasm resulting in host cell lysis which releases mature viral particles. The sequence was generated by mass spectrometry by matching peptides across a range of search engines including X! Tandem, Comet, Tide, Sage and PeptideShaker. The protein sequence was further subjected to BLASTp analysis in order to determine conservation sites (Altschul *et al*., 1997). Multiple sequence alignments with the top five BLASTp results revealed that the sequence is homologous and highly conserved across T4 bacteriophages (Figure 3.2B). Bioinformatics analysis revealed 13 peptides that constitute the endolysin protein, which has a molecular weight of 18.62 kDa (Table 3.2). The current study also determined the degree of protein deamidation, oxidation or carbamidomethylation of

endolysin peptides as it relates to the degradation of the protein. The identification of deamidated peptides is crucial for understanding protein dynamics and ensuring the quality of biopharmaceuticals (Hao and Sze, 2014). Deamidation of asparagine was noted in peptides 4 (N;3,4), 6 (N;1), 7 (N;8,9,12), 9 (N;5) and 11 (N;5). Oxidation of methionine was noted in peptides 2 (M;19), 9 (M;6) and 11 (6). Carbamidomethylation of cysteine was noted in peptides 2 (14), 8 (C;14), 9 (C;1), 11 (C;1), 12 (C;6) and 13 (C;2). Modification of amino acids may occur spontaneously in proteins and the evaluation of these modifications provides insight into the stability and biological activity of the protein both *in vitro* and *in vivo*. However, since the protein sample was digested with trypsin it is challenging at this stage to determine whether these modifications were as a result of natural or artificial degradation without further analyses.

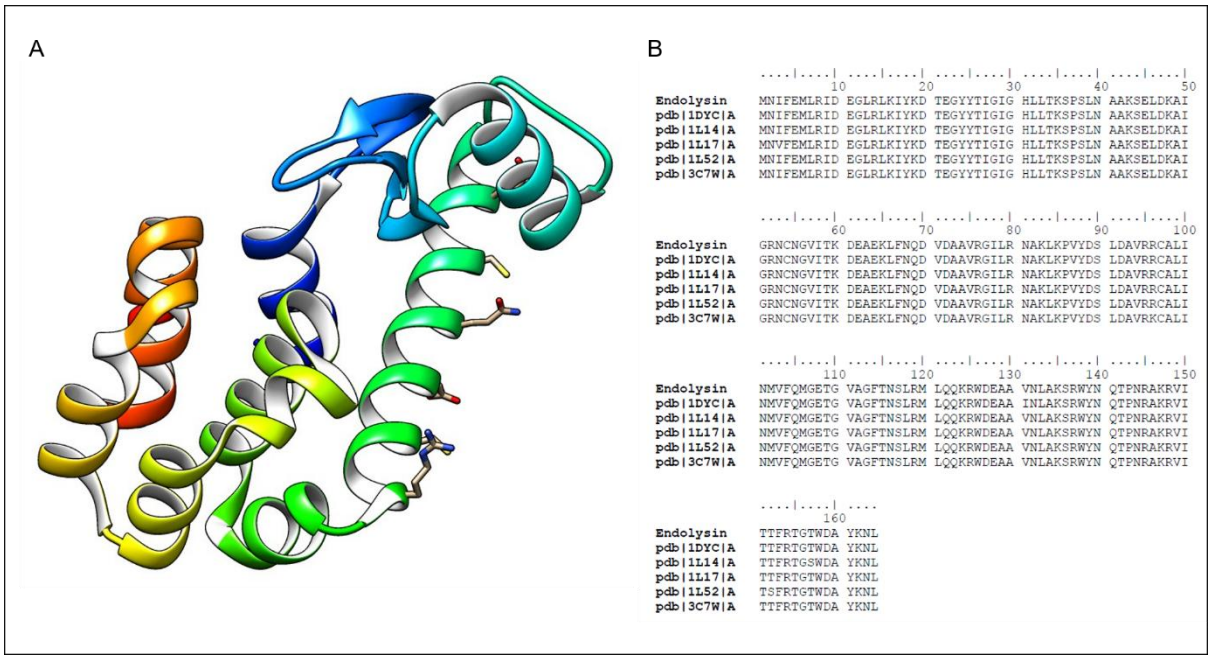

Figure 3.2: Predicted structure of endolysin protein from *Escherichia* bacteriophage T4 detected by liquid chromatography. (A) Structure was homologous with the T4 lysozyme from the Protein Data Bank (PDB ID; 8TAT). (B) Multiple sequence alignments of endolysin from the current experiment with top five proteins returned by BLASTp revealing a homologous and highly conserved amino acid sequence.

Table 3.2: Peptides and peptide modifications associated with the endolysin protein detected in *Escherichia* bacteriophage T4.

Peptide Sequence	Modified Sequence	Position	Amino Acids Before	Amino Acids After
MNIFEMLRIDEGLRL	NH2-MNIFEMLRIDEGLRL-COOH	1		KI
LKPVYDSLDAVRRCALINMVFQMGETGVAG	NH2-LKPVYDSLDAVRRC<cmm>ALINM<ox>VFQMGETGVAG-COOH	84	AK	FT
DTEGYYTIGIGH	NH2-DTEGYYTIGIGH-COOH	20	YK	LL
MLQQKRWDEAA	NH2-MLQ<deam>Q<deam>KRWDEAA-COOH	120	LR	VN
DTEGYYTIGI	NH2-DTEGYYTIGI-COOH	20	YK	GH
NAKLKPVY	NH2-N<deam>AKLKPVY-COOH	81	LR	DS
LAKSRWYNQTPNRAK	NH2-LAKSRWYN<deam>Q<deam>TPN<deam>RAK-COOH	133	VN	RV
LKPVYDSLDAVRRCALINM	NH2-LKPVYDSLDAVRRC<cmm>ALINM-COOH	84	AK	VF
CALINMVF	NH2-C<cmm>ALIN<deam>M<ox>VF-COOH	97	RR	QM
NIFEMLRIDEGLR	NH2-NIFEMLRIDEGLR-COOH	2	M	LK
CALINMVFQ	NH2-C<cmm>ALIN<deam>M<ox>VFQ-COOH	97	RR	MG
AIGRNCNG	NH2-AIGRNC<cmm>NG-COOH	49	DK	VI
NCNGVITKDEAEKLFNQDVDAAVR	NH2-NC<cmm>NGVITKDEAEKLFNQDVDAAVR-COOH	53	GR	GI

Ox: Oxidation; Deam: Deamidation of N; Cmm: Carbamidomethylation of C

3.3.2. Separation of bacteriophage T4 proteins and identification of active fractions

Proteomic analysis aided in the determination of the molecular weight of the endolysin from bacteriophage T4 (18.62 kDa). This guided gel filtration targeted towards its isolation. *Escherichia* bacteriophage T4 was propagated with *E. coli* and total protein extracted via ammonium sulphate precipitation. Separation of bacteriophage proteins via gel filtration yielded 50 fractions that were subjected to the spot assay. Fractions 19 – 24 displayed clear efficacy against *E. coli* compared to the earlier and later fractions (Figure 3.3). Total soluble protein of active fractions as determined by the Bradford assay, ranged between 10.93 to 11.36 mg/ml.

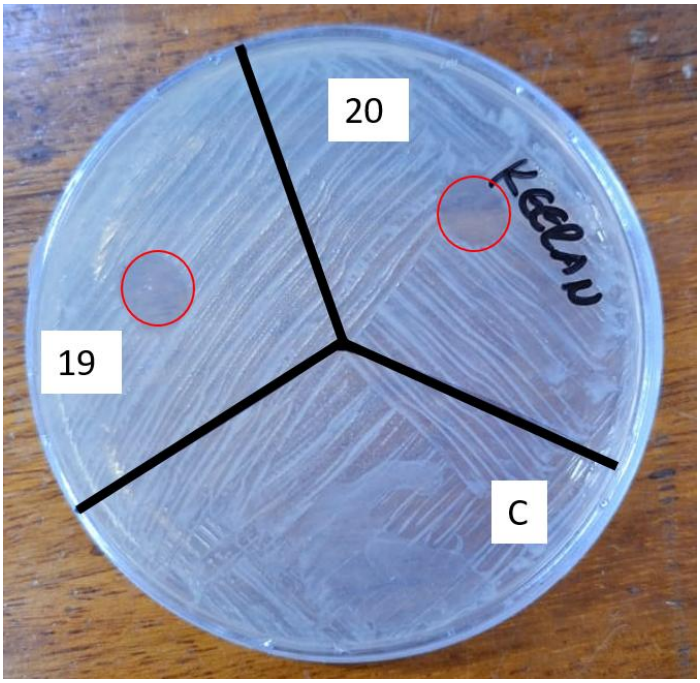

Figure 3.3: Antibacterial spot-on assay on nutrient agar with *Escherichia coli* exhibiting transparent spots (red circles) indicative of bactericidal activity by selected fractions (fractions 19 and 20) as compared to the buffer control (C).

Bactericidal activity of fractions 19 – 24 derived from Bacteriophage T4 against *E. coli* was also evaluated in a microdilution assay, the results of which are presented in Table 3.3.

Table 3.3: The minimum inhibitory concentrations (MIC) of protein derived from bacteriophage T4 required to inhibit the growth of *Escherichia coli*.

Antibacterial MIC (µg/ml)	
Fraction	***E. coli***
19	43.70 ± 0.01
20	43.90 ± 0.01
21	42.80 ± 0.01
22	43.03 ± 0.01
23	42.78 ± 0.01
24	43.00 ± 0.01

Fractions 19 to 24 displayed similar MIC values against *E. coli* ranging between 42.78 and 43.90 µg/ml. While it has been suggested that pure isolated compounds possessing MIC values of less than 1 mg/ml are considered potentially useful (Fabry *et al.,* 1998; Gibbons, 2005), the useful concentration limits of antimicrobial proteins have not been established. However, antimicrobial proteins range in activity from 1-100 µg/ml. The proteins isolated from bacteriophage T4 in this study exhibited antimicrobial activity against *E. coli* that is at the lower end of the range, underscoring their potential for therapeutic application. However, further research is required to determine their efficacy against BPPL associated pathogenic bacteria. Furthermore, given the concern over their stability highlighted earlier in this chapter, methods to improve the stability of proteins to aid in drug delivery needs to be researched.

CHAPTER 4: ANTIMICROBIAL ACTIVITY OF BACTERIOPHAGE T4 PROTEIN FRACTIONS AGAINST BPPL PATHOGENIC BACTERIA

4.1. Background Information

Infectious diseases caused by pathogenic bacteria continue to be a major cause of mortality, despite the significant resources and research dedicated to developing effective treatment strategies. For instance, in 2019, infection with *S. aureus* was the leading cause of death in 135 countries (Ikuta *et al*., 2022). Prolonged illness, increased risk of complications, limited treatment options and increased risk of mortality amongst vulnerable populations are a few of the many issues surrounding such infections. Thus, the search for and implementation of new antimicrobials is crucial in mitigating the impact of bacterial infections. Bacteriophage T4 protein fractions reported in **Chapter 3** displayed antimicrobial activity towards *E. coli*. The current chapter primarily focuses on extending the work to bacteria associated with the BPPL developed by the World Health Organization. Therefore, protein fractions were evaluated for their ability to inhibit the growth of *K. pneumoniae*, *S. aureus*, *E. faecalis*, and *P. mirabilis*. Furthermore, the stability of protein therapeutics is a critical concern due to factors pertaining to their safety, efficacy and shelf life. Liposomal encapsulation of therapeutics has been recognized as a promising drug delivery system, capable of protecting the drug of interest from degradation while also improving the drug's efficacy (Salahshoori *et al*., 2024). Liposomes consist of several different lipids which link together to form a spherical structure with an aqueous center. The presence of the aqueous phase makes liposomes a desirable mode of encapsulation and delivery of several molecules such as, water soluble drugs, antimicrobial proteins as well as vitamins and antioxidants (Taylor *et al*., 2005; Mozafari *et al*., 2006; Date *et al*., 2007; Teixeira *et al*., 2008). Consequently, bacteriophage T4 proteins were encapsulated within a liposomal layer and assessed for their capacity to inhibit bacterial growth, aiming to establish a reliable method for enhancing the stability and efficacy of bacteriophage proteins.

4.2. Methodology

4.2.1. Purification and preparation of active fractions

To obtain a pure isolate of bacteriophage proteins, the active fractions derived from **Section 3.3.2** of the current thesis were pooled and the protein was precipitated by saturating up to 85% with ammonium sulfate. The solution was incubated at 4°C overnight following which it was centrifuged at 10 000 rpm for 1 h at 4°C. The resultant protein pellet was collected and dissolved in 5 ml phosphate buffer and separated by gel filtration with Sephadex G-50 (Sigma-Aldrich, Germany) as the stationary phase. Fifty 1 ml fractions were collected. The absorbance of these fractions was recorded at 280 nm, and those fractions that displayed a distinct peak were selected for further analysis.

4.2.2. Estimation of protein content

The protein content of the fractions was quantified using a modified Bradford Protein Assay. (Bradford, 1976). Briefly, 50 µl of water was added to each well in a 96-well microplate. Subsequently, 50 µl of each fraction was added to the top well and the fractions were serially diluted twice. Following this, 50 µl of the Bradford Reagent was added and the absorbance was measured at 595 nm. A calibration curve was developed using bovine serum albumin to determine the concentration of protein in each fraction. The analysis of protein content facilitated the determination of the minimum concentrations required to inhibit the growth of the various bacterial strains evaluated in this study.

4.2.3. Preparation of liposomes

Liposomes were prepared using the thin film hydration method (Zhang, 2016). L-α-phosphatidylcholine (16 µM), stearylamine (18 µM) and cholesterol (9 µM) were dispensed into a round bottom flask to which 1 ml of chloroform was added. The lipid and chloroform mixture were placed on an orbital shaker allowing the chloroform to evaporate leaving behind a thin film of lipids on the surface of flask. The lipid film was then left to dry further under a drying fan. Once the lipid film was dry, 1 ml of phosphate buffer containing the bacteriophage protein was added to the flask and swirled for 30

min on an orbital shaker. A modified Bradford Protein Assay was used to determine the protein content within the liposomes. The liposome solution (200 µl) was centrifuged at 1000 rpm for 15 min. The supernatant was removed and replaced with PBS. This procedure was repeated three times to remove any un-encapsulated protein. Triton-X 100 (0.25 µl) was added, and the solution was vortexed vigorously to break open the liposome. Determination of protein followed the procedure as outlined in **Section 4.2.2** of this thesis.

4.2.4. Transmission electron microscopy

Transmission electron microscopy (TEM) was employed to confirm successful formation of the liposomes. Liposomes were negatively stained using uranyl acetate and a droplet of the liposome suspension was placed onto Parafilm®. A copper grid coated with Formvar was inverted onto the droplet to allow particles to attach via electrostatic forces. The copper grid was allowed to rest for 5 min and was wicked dry with Whatman filter paper. Subsequently, a drop of uranyl acetate (2%) was placed onto Parafilm® and the grid was inverted on the drop for 30 s, excess liquid was wicked away with Whatman filter paper and the grid was allowed to dry before viewing under the TEM. Samples were viewed under 5000x magnification.

4.2.5. Antibacterial activity of bacteriophage T4 protein and T4 protein loaded liposomes

The antimicrobial activity of the bacteriophage T4 protein and T4 protein loaded liposomes was determined using the microdilution bioassay described by Eloff (1998) with slight modifications. Overnight cultures (incubated at 37°C in a water bath with an orbital shaker) of *K. pneumonia*, *E. faecalis*, *P. mirabilis*, *S. aureus* and *E. coli* was diluted with 20 ml sterile Mueller-Hinton (MH) (Sigma-Aldrich, Germany) broth (200 µl in 19.8 ml) in accordance with the McFarland standard. In a 96-well microplate, either 100 µl of the bacteriophage protein solution (maintained in PBS) or 50 µl of the liposomal protein were serially diluted two-fold with 100 µl and 50 µl of sterile distilled water, respectively. Subsequently, 100 µl or 50 µl of the three bacterial cultures was added to each well containing either the bacteriophage protein or the liposomal protein, respectively. A two-fold serial dilution of neomycin (Sigma-Aldrich, Germany) (0.1 mg/ml), an aminoglycoside effective against Gram-positive and Gram-negative

bacteria (Kumar *et* al., 2020), was used as a positive control, while sterile distilled water was used as the negative control. The plates were covered with Parafilm® and incubated at 37°C for 24 h. Bacterial growth was determined by the addition of 50 µl of *p*-iodonitrotetrazolium chloride (INT; 0.2 mg/ml) (Sigma-Aldrich, Germany).

4.2.6. Prediction of molecular interactions using molecular modeling

Bacteriophage T4 proteins displayed promising activity against *S. aureus*. Molecular modeling was employed in order to determine potential molecular interactions that may be responsible for this activity. The high-resolution crystal structures of the penicillin binding proteins 1 – 4 (PDB IDs: 1TVF, 3VSL, 6H5O and 7O49) were downloaded from the Protein Data Bank along with the endolysin (PDB ID: 8TAT) identified by proteomics in **Section 3.3.1** of the current thesis. Protein structures were prepared for docking by removing all non-native and ligand molecules and polar hydrogen atoms and Kollman charges were added. Proteins were energy minimized using the Gromos 96 force field and the prepared proteins were submitted for molecular docking using the GRAMMX Protein-Protein Interaction Server (Tovchigrechko and Vakser, 2006). Binding energy scores were assessed with PRODIGY HADDOCK and the molecular interactions were determined with DimPlot (Wallace *et al*., 1996; Vangone and Bonvin, 2015).

4.2.7. Data analysis

Unless otherwise specified, all experiments were conducted twice with three replicates each. Data pertaining to antimicrobial activity of bacteriophage T4 protein and T4 protein loaded liposomes were subjected to an independent samples T-test.

4.3. Results and Discussion

4.3.1. Preparation of bacteriophage proteins and liposomal encapsulation

Finding solutions to the public health challenges posed by the spread of pathogenic and multidrug-resistant (MDR) bacteria is critical, given the threat these issues pose to achieving the United Nations SDGs. The current study aimed to tackle this challenge by assessing the feasibility of using phage proteins to combat the threat posed by pathogenic bacteria. Proteins were precipitated with ammonium sulfate (saturated up to 85%) and separated by gel chromatography using Sephadex G-50

super fine powder. The reason for implementing a second purification step was to obtain a more concentrated protein extract. The selection of the stationary phase for this separation was influenced by proteomic analysis, which indicated that the endolysin from bacteriophage T4 had a molecular weight of 18.62 kDa, falling within the range of Sephadex G-50. A microdilution assay indicated that out of the 50 fractions collected, fractions 9 to 18 demonstrated strong activity against the preliminary screening organism, *E. coli*. The results of this experiment are presented in Table 4.1. Fractions 14 and 15 displayed the most potent activity against *E. coli* and were selected for further analysis.

Table 4.1: Total protein content and antimicrobial activity of bacteriophage T4 fractions against *Escherichia coli*.

Fraction	Total Protein (mg/ml)	Minimum Inhibitory Concentration against *Escherichia coli* (µg/ml)
9	0.63 ± 0.09	19.61 ± 0.01
10	1.08 ± 0.12	16.92 ± 0.01
11	1.14 ± 0.08	8.90 ± 0.01
12	1.29 ± 0.14	5.00 ± 0.01
13	1.30 ± 0.11	10.00 ± 0.01
14	1.38 ± 0.14	**3.30 ± 0.01**
15	1.54 ± 0.08	**3.50 ± 0.01**
16	0.60	6.71
17	1.15	8.97
18	0.87	13.66

Fractions 14 and 15 were combined and liposomes were generated from 1 ml of this solution. Liposome integrity was examined through TEM analysis, as illustrated in Figure 4.1. Round to oval vesicles were nano-sized, ranging from 0.37 to 0.73 µm and presented as agglomerated clusters or individual liposomes (Figure 4.1A and B, respectively). Empty liposomes were also visualized. The encapsulation of the protein was confirmed by rupturing 200 µl of liposomes with 0.25 µl of Triton X-100 (Sigma-

Alrich) and performing the Bradford protein assay on the resulting lysate. Approximately 0.76 mg of protein was contained within 1 ml of the liposome solution. Encapsulation efficiency was assessed based on the initial protein concentration, resulting in an efficiency of 52.05 ± 2.4%.

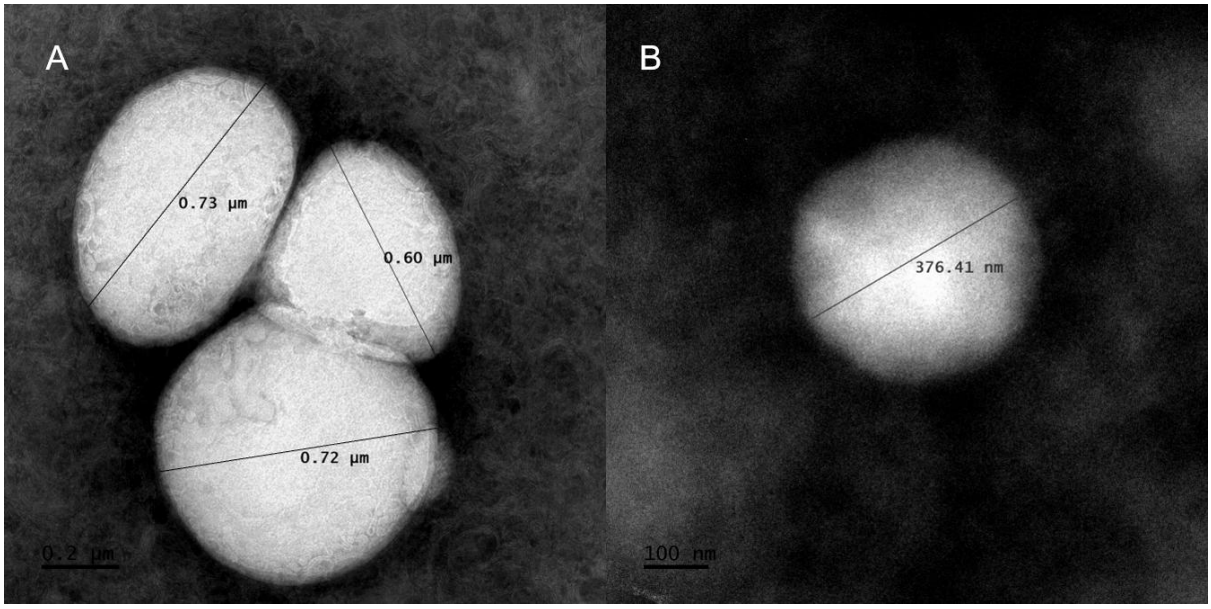

Figure 4.1: Transmission electron microscopy (TEM) micrographs of liposomes. (A) Optimum agglomerated bacteriophage T4 protein loaded ovate liposome (0.60 – 0.73 µm, scale bar; 0.2 µm). (B) Optimum non-agglomerated bacteriophage T4 protein loaded circular liposome (37.41 µm, scale bar; 0.1 µm).

4.3.2. Antimicrobial activity of bacteriophage T4 proteins and T4 protein loaded liposomes

The antimicrobial activities of bacteriophage T4 proteins and T4 protein loaded liposomes are presented in Table 4.2. Bacteriophage T4 proteins demonstrated varying levels of effectiveness in inhibiting the growth of the bacteria assessed in this study. For instance, while the protein was able to inhibit the growth of *S. aureus* at a concentration of 12.87 µg/ml, it was incapable of influencing the growth of *E. faecalis*, *P. mirabilis* and *K. pneumoniae* at the highest concentration tested (205.68 µg/ml). Notably, in every instance, the liposomes loaded with T4 protein were more effective at inhibiting the growth of the bacteria.

Table 4.2: Antimicrobial activity of bacteriophage T4 proteins and T4 protein loaded liposomes.

	Minimum Inhibitory Concentrations		
	Bacteriophage T4 Protein (µg/ml)	**T4 Protein Loaded Liposome (µg/ml)**	**Neomycin (µg/ml)**
Staphylococcus aureus	$12.87 \pm 0.01_a$	$5.99 \pm 0.01_b$	$0.8 \times 10^{-3} \pm 0.01$
Enterococcus faecalis	$>205.68 \pm 0.01_a$	$5.99 \pm 0.01_b$	$1.7 \times 10^{-3} \pm 0.01$
Proteus mirabilis	$>205.68 \pm 0.01_a$	$95.90 \pm 0.01_b$	$1.4 \times 10^{-3} \pm 0.01$
Klebsiella pneumoniae	$>205.68 \pm 0.01_a$	$192.00 \pm 0.01_b$	$1.6 \times 10^{-3} \pm 0.01$

Different letters represent significant differences at a significance level 0.05.

Gram-positive *S. aureus* is a common commensal bacterium that thrives on human skin and mucosal membranes. However, in immunocompromised individuals, it can cause severe soft tissue and skin infections (Portilla *et al*., 2020). The emergence of methicillin-resistant *S. aureus* (MRSA) is particularly concerning, as it can lead to life-threatening illnesses and is often associated with high treatment failure rates due to multidrug resistance. Moreover, vancomycin, the last resort for treatment, has significant limitations, including nephrotoxicity and the risk of resistance development (Portilla *et al*., 2020; Brown *et al*., 2021). Thus, the ability of bacteriophage T4 proteins to interact with and inhibit the proliferation of *S. aureus* in the current study, offers a promising alternative for combating this pathogen. Similarly, numerous studies (Loeffler *et al*., 2001; Nelson *et al*., 2001; O'Flaherty *et al*., 2005; Lai *et al*., 2011) have demonstrated the efficacy of exogenously applied, purified bacteriophage endolysins in combating Gram-positive bacteria. However, the bacteriophage T4 protein in the present study failed to exhibit any activity against Gram-positive *E. faecalis* at the highest concentration tested, this is likely due to the presence of a capsular polysaccharide layer. Many bacterial organisms, including *E. faecalis*, produce a capsular polysaccharide layer that surrounds the bacterium, shielding it from toxins and the host immune response, thereby enhancing its virulence (Ganesh *et al*., 2017). The presence of a capsular polysaccharide layer may thus have prevented the

attachment of bacteriophage T4 proteins limiting its lytic activity. However, liposomes loaded with the T4 proteins were capable of inhibiting growth of *E. faecalis* at a concentration of 5.99 µg/ml. This suggests that the liposome may be a promising solution to overcoming the bacterial capsule that constitutes one of the key mechanisms by which many bacterial species evade antimicrobial drugs.

The current study assessed the ability of bacteriophage T4 proteins to inhibit two Gram-negative bacterial strains; *P. mirabilis* and *K. pneumoniae*. *Proteus mirabilis* has been implicated in a variety of infections including respiratory and wound infections and is also a frequent cause of catheter-associated urinary tract infections (Abdulkhadhim *et al.,* 2024). *Klebsiella pneumoniae* is primarily found in the respiratory tract and intestines of humans and an imbalance may result in the development of nosocomial infections that could lead to death if left untreated (Li *et al*., 2024). No activity was noted against either of these strains at the highest concentration at which the bacteriophage T4 protein was tested. However, T4 protein loaded liposomes returned reasonable MIC values against *P. mirabilis* (95.90 ± 0.01 µg/ml) and *K. pneumoniae* (192 ± 0.01 µg/ml). Both *P. mirabilis* and *K. pneumoniae* possess a range of sophisticated mechanisms by which they evade antimicrobial drugs including biofilm production. Biofilm producing bacteria have been noted to be able to produce antibiotic degrading enzymes that may have reduced the stability of the bacteriophage T4 protein in the current study (Ciofu and Tolker-Nielsen, 2010). The bacteria also deploy receptor proteins that have low binding affinity with antibiotics which may also have contributed to the limited lytic activity noted in this study (Ciofu and Tolker-Nielsen, 2010). However, T4 protein loaded liposomes have overcome these barriers and protected the protein from decay and thus displayed significantly greater antimicrobial activity. Similarly, Bai *et al*. (2019) achieved a significant reduction in viable bacterial cells of *Salmonella typhimurium* and *E. coli* with the exogenous application of liposomes containing BSP16Lys an endolysin isolated from a *S. typhimurium* bacteriophage, achieving a 2.2-log CFU/ml and 1.6-log CFU/ml reduction in bacterial cell numbers, respectively. The antimicrobial activity of liposomes containing lytic bacteriophage proteins was further demonstrated by Morais *et al* (2022). Lytic enzymes pa7 and pa119 from a *Pseudomonas aeruginosa* bacteriophage were encapsulated within a liposome and applied exogenously. The liposome formulation was able to kill *P. aeruginosa* within 48 h (Morais *et al*., 2022).

Notably, the T4 protein-loaded liposome developed in this study demonstrated a faster antimicrobial effect, eradicating two Gram-negative organisms within 24 h, whereas the previous study required 48 h to achieve similar results.

The results presented here demonstrate the capability of T4 protein loaded liposomes to interact with and inhibit the growth of important bacteria linked to the BPPL as identified by the WHO, providing a pathway for the development of novel drugs aimed at combating these devastating bacteria.

4.3.3. Prediction of molecular interactions between bacteriophage T4 protein and *S. aureus*

Bacteriophage T4 proteins assessed in the current study displayed encouraging activity against *S. aureus* in an *in vitro* microdilution assay. *In silico* molecular modeling experiments were conducted to explore potential interactions between these proteins and *S. aureus*, providing insight into possible mechanisms of action. For this work, the endolysin from the bacteriophage T4 identified via mass spectrometry was modeled against the penicillin binding proteins (PBP) 1 - 4 from *S. aureus*. The results of this experiment are presented in Table 4.3. The endolysin/PBP3 complex returned the strongest binding energy score (-29.6 kcal/mol), followed by the endolysin/PBP2 complex (-29.1 kcal/mol), the endolysin/PBP1 complex (-24.4 kcal/mol) and the endolysin/PBP4 complex (-21.6 kcal/mol), respectively. Protein-protein interactions are responsible for most processes that are required for optimum cellular metabolism (Kastritis *et al*., 2014). The binding affinity is a metric that determines whether proteins can form a complex relation to their concentration. Furthermore, the interaction between participants in these complexes was also quantified by the dissociation constant (*K*d) which is an important parameter used to measure protein-protein interactions (Jiang *et al*., 2019). The endolysin/PBP2 complex returned the strongest interaction at 4.60 x 20^{-22} M, representative of the concentration of the endolysin at which half of the PBP2 target sites are occupied (Table 4.3).

Table 4.3: Binding affinity and Kd prediction derived from PRODIGY HADDOCK for the endolysin and penicillin binding protein (1 - 4) complexes

Receptor	ΔG (kcal/ mol)	Kd (M) at 25 °C	ICs Charged-Charged	ICs Charged-Polar	ICs Charged-Apolar	ICs Polar-Polar	ICs Polar-Apolar	ICs Apolar-Apolar
PBP1	-24.4	1.20×10^{-18}	28	36	57	4	53	47
PBP2	-29.1	4.60×10^{-22}	28	41	90	14	64	47
PBP3	-29.4	2.90×10^{-22}	63	41	94	11	48	44
PBP4	-21.6	1.30×10^{-16}	37	38	67	7	31	20

PBP: Penicillin Binding Protein; ICs: Interfacial Contacts.

The PBPs are transpeptidases or carboxypeptidases that are required for the formation of the peptidoglycan layer in *S. aureus*, *Enterococci* spp. and *Streptococcus* spp. (Zapun *et al*., 2008). Transpeptidation or the cross-linking of stem peptides (in addition to glycosylation) incorporates peptidoglycan precursors into the peptidoglycan layer (Bertonha *et al*., 2023). Inhibition of transpeptidation associated cross-linking results in degeneration of the peptidoglycan layer and cell lysis (Macheboeuf *et al*., 2006). Given that PBPs are the only proteins capable of catalyzing transpeptidation reactions, they represent an attractive target for drug development. The activity presented by bacteriophage T4 proteins may be directly related to their affinity towards the PBPs, disrupting cell wall formation. This was corroborated by the strong affinity demonstrated by the endolysin towards the PBPs in the present study.

The catalytic site of PBP1 from *S. aureus* comprises three motifs: the SXXK region, which spans from Ser314 to Lys317; the (S/Y) XN motif, located from Ser368 to Asn370 and the (K/H) (S/T) G motif, located from Lys513 to Thr516 (Zapun *et al*., 2008; Bon *et al*., 2024). Small molecule antibiotics inhibit catalytic activity of the enzyme by binding to the positively charged active site pocket and reducing its catalytic efficiency (Zapun *et al*., 2008). However, the endolysin protein, which has a molecular weight of 18.62 kDa and consists of 162 amino acids, is too large to enter the active site pocket. Thus, the molecular interactions between the endolysin and the PBPs were determined through blind docking in which the entire surface of the PBPs was explored to identify potential binding interactions. The molecular interactions involved in the formation of the endolysin/PBP1 complex are illustrated in Figure 4.2.

The endolysin protein was observed to interact with several amino acid residues of the pedestal domain of the PBP1 located from Met1 to Gly237. The pedestal domain is characterized by three crucial subdomains that anchor and localize the PBP to the inner membrane, providing a site for shape, elongation, division and sporulation (SEDS) proteins to bind (Bon *et al*., 2024). In addition to cross-linked stem peptides, the peptidoglycan layer of *S*. *aureus* is composed of polymerized disaccharide subunits mediated by SEDS proteins through glycosylation (Bertonha *et al*., 2023). The affinity of the endolysin to residues associated with SEDS protein binding suggests a role for the endolysin as an inhibitor of glycosylation of peptidoglycan units.

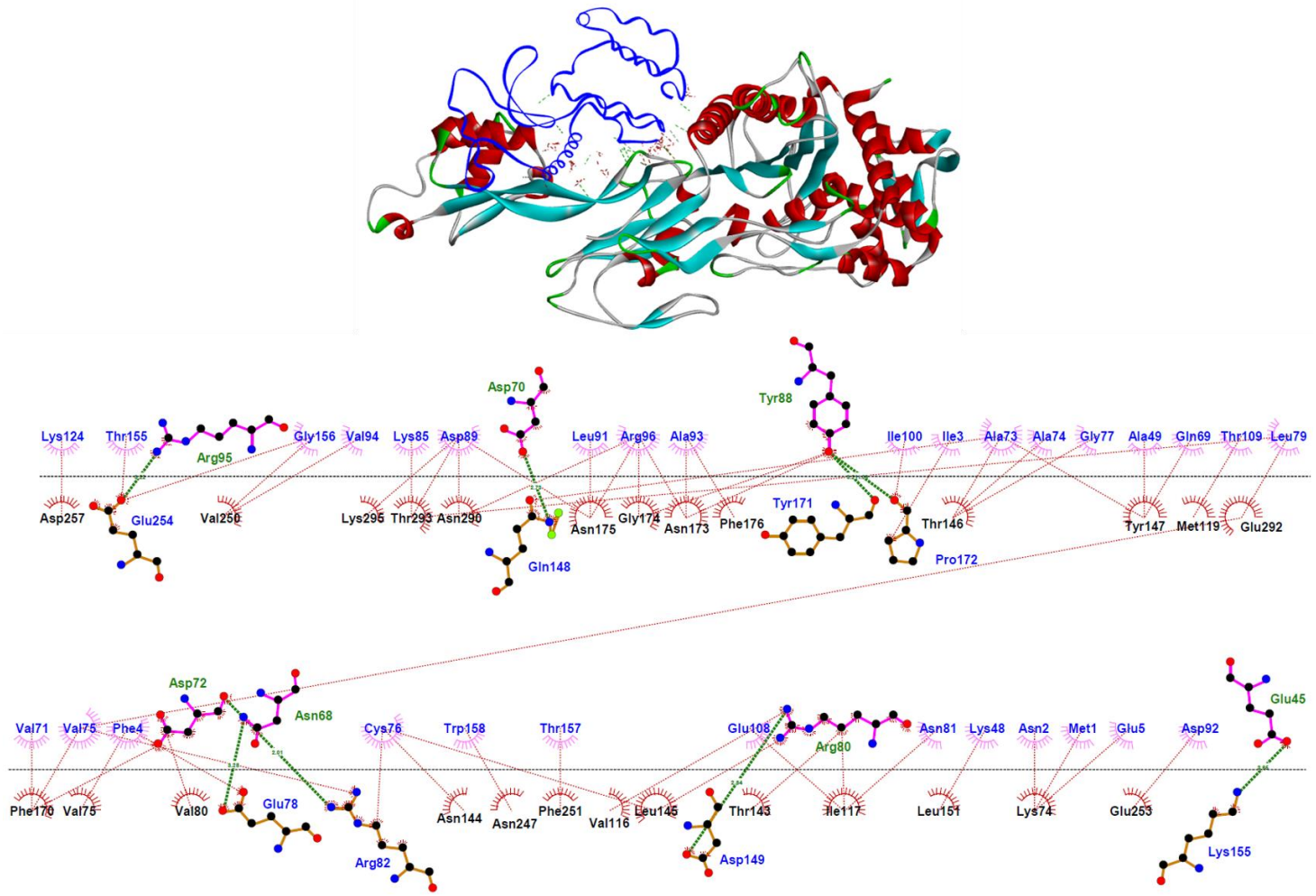

Figure 4.2: The predicted mode of interaction between the amino acid residues of the endolysin from bacteriophage T4 (represented as a blue flat ribbon) and those of the penicillin-binding protein 1 from *Staphylococcus aureus* (shown as a teal/red ribbon). Hydrogen bonds are depicted with thick green dotted lines, while hydrophobic interactions are represented by thin red dotted lines.

Out of the four PBPs produced by *S. aureus*, only PBP1 and PBP2 are essential for peptidoglycan synthesis, with the PBP2 serving as the primary synthase (Wacnik *et al*., 2022). The molecular interactions involved in the formation of the endolysin/PBP2 complex are illustrated in Figure 4.3. The PBP2 from *S. aureus* is composed of an anchor domain, a transpeptidase domain and a transglycosylase domain. It is interesting to note that the endolysin protein exhibited multiple interactions with amino acids from these domains (Figure 4.3). The ability of the endolysin to interact with these residues suggests a potential mechanism by which the protein could inhibit essential mechanisms involved in bacterial peptidoglycan synthesis.

Similarly, the inhibition of PBPs by small molecules and peptides has now been well documented. For instance, modifications to Avibactam, resulted in the development of compounds that displayed an affinity towards PBPs from *E. coli* (King *et al*., 2016). Similar activities were also demonstrated by the heterocyclic 4-quinolones against PBPs (Shilabin *et al*., 2012). However, because bacterial pathogens inherently possess the ability to develop resistance to new antibiotics, the search for and development of novel drugs is under pressure. The results presented here provide evidence for the use of bacteriophage proteins to target PBPs with the potential to save millions of lives.

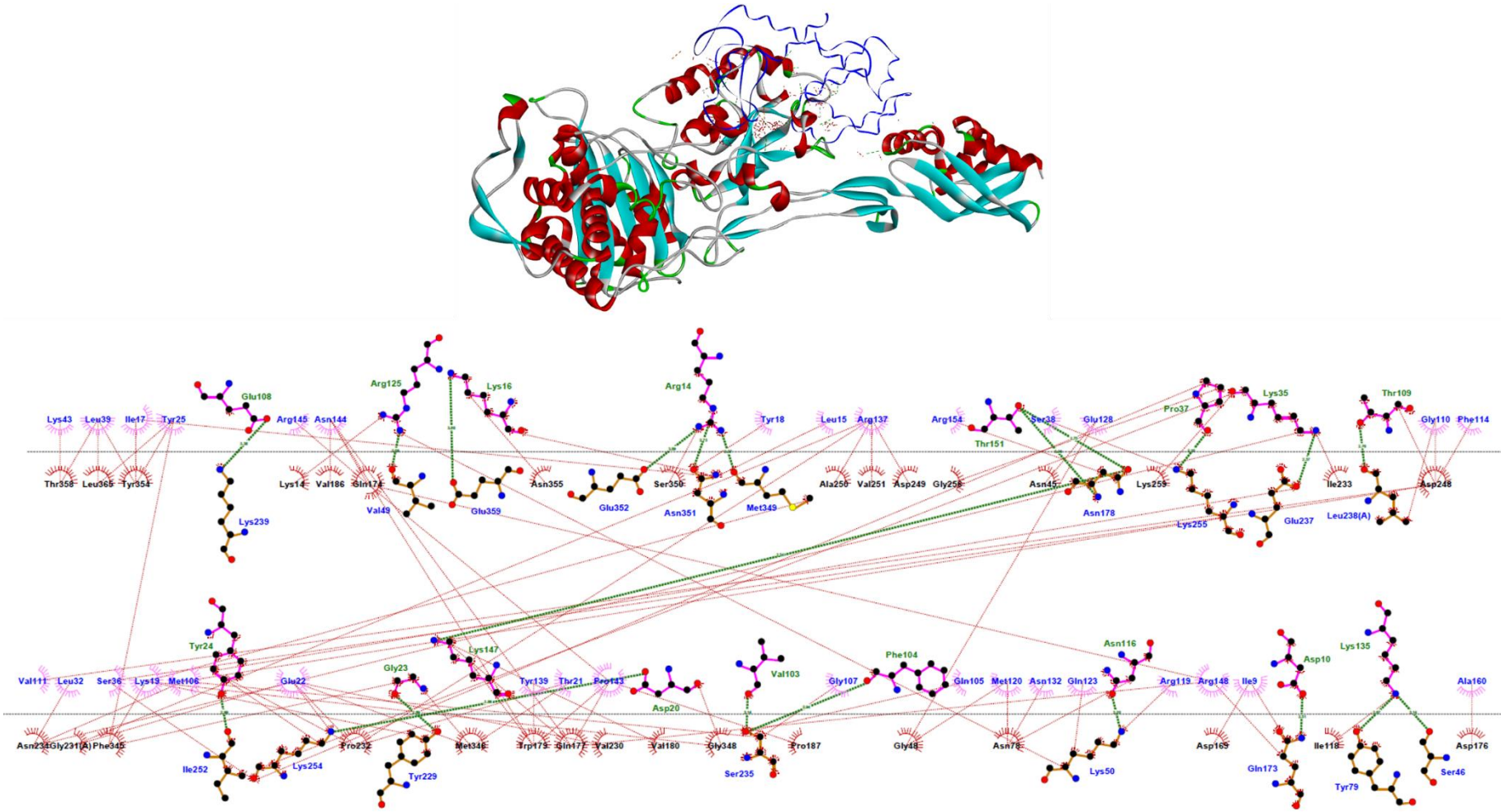

Figure 4.3: The predicted mode of interaction between the amino acid residues of the endolysin from bacteriophage T4 (represented as a blue flat ribbon) and those of the penicillin-binding protein 2 from *Staphylococcus aureus* (shown as a teal/red ribbon). Hydrogen bonds are depicted with thick green dotted lines, while hydrophobic interactions are represented by thin red dotted lines.

CHAPTER 5: GENERAL CONCLUSIONS AND RECOMMENDATIONS

Pathogenic bacteria remain significant concern for global public health, affecting vulnerable populations such as neonates, young children, and immunocompromised individuals (Ikuta et al., 2022). Bacterial infections consistently rank among the leading causes of mortality, exemplified by sepsis, which accounted for 20% of the total global mortality rate in 2017. Prominent pathological organisms contributing to sepsis-related fatalities globally include *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Ikuta et al., 2022). Given this significant threat, the World Health Organization (WHO) has highlighted a critical need for novel therapeutic regimens to combat these priority pathogens.

In the present study, proteins from the *Escherichia* bacteriophage T4 were extracted and analyzed for their proteomic characteristics to gain deeper insights into the protein structures that may confer antimicrobial activity. The proteins were encapsulated within a liposome to determine if encapsulation enhances the antimicrobial activity against Gram-negative and Gram-positive bacteria. The endolysin protein demonstrated varying levels of effectiveness in inhibiting the growth of the bacteria assessed in this study. For instance, while the protein was able to inhibit the growth of *S. aureus* at a concentration of 12.87 µg/ml, it was incapable of influencing the growth of *E. faecalis*, *P. mirabilis* and *K. pneumoniae* at the highest concentration tested (205.68 µg/ml). Notably, in every instance, the liposomes loaded with T4 protein were more effective at inhibiting the growth of the bacteria. The findings presented in this study demonstrate the capability of T4 protein loaded liposomes to interact with and inhibit the growth of important bacteria linked to the BPPL as identified by the WHO, providing a pathway for the development of novel drugs aimed at combating these devastating bacteria.

Molecular modeling techniques were employed to decipher the molecular interactions between the bacteriophage T4 endolysin and the peptidoglycan of *S. aureus*, particularly concerning their PBPs, which are critical targets for the development of new drugs. The endolysin protein exhibited strong affinity towards all four PBPs of *S. aureus* corroborating the results of the microdilution assays. Further analysis revealed

that the endolysin binds to important residues that are involved in the catalytic activity of PBP1 and PBP2 suggesting that it may be capable of inhibiting transpeptidation and transglycosidation, resulting in the breakdown of the bacterial cell wall.

The results of the present study demonstrate the potential of employing bacteriophage proteins as therapeutic agents against pathogenic bacteria as it presents a solution to the current state of the art. However, the findings of this study suggest that the bacteriophage T4 proteins and bacteriophage T4 protein loaded liposomes offer attractive antimicrobial properties. Further research is still required to properly evaluate its potential to replace the current antibiotics. For instance;

- *in vitro* evaluation on a wider variety of bacterial species may provide more perspective on the proteins ability to be used as a broad-spectrum antimicrobial.
- Combination studies with current antibiotics should be investigated to demonstrate synergy as well as reduce development of resistance to current antibiotics.
- Endolysin engineering studies can be carried out to improve stability and antimicrobial activity.
- *In vivo* studies can be conducted on animal models to evaluate various delivery methods (topical, oral or intravenous), while studies on pharmacokinetics, half-life and possible side effects are also required.
- Furthermore, while only 130 of the 300 *Escherichia* bacteriophage T4 proteins have been characterized, there exists opportunities for the discovery of new antimicrobials from this organism.

While this study presents promising findings that support the potential use of bacteriophage endolysins as novel antimicrobial agents, the study did have some limitations. The lack of funding restricted the isolation of a pure endolysin extract through protein electrophoresis. Furthermore , the extraction process did not yield enough endolysin to perform invitro studies on the stability of the endolysin and the liposomal encapsulated endolysin.

References

Abdulkadim, H. M., Abdullah, A. S. R. M. 2024. Evaluation of bacteriophage effect against multidrug-resistant *Proteus mirabilis* isolated from different clinical samples. *Research Journal of Biotechnology*, 9: 11.

Abraham, E. P., Chain, E. 1940. An enzyme able to destroy penicillin. *Nature*, 146(3713): 837 – 837.

Adams, M. D., Nickel, G. C., Bajakscovzian, S., Lavender, H., Murthy, A., Jacobus, M. R., Bonomo, R. A. 2009. Resistance to Collistin in *Acinetobacter baumannii* associated with mutations in the Pmr AB two-component system. *Antimicrobial Agents and Chemotherapy,* 53(9): 3628 – 3634.

Ajuebor, J., Mcauliffe, O., O'Mohony, J., Ross, R. P., Hill, C., Coffey, A. 2018. Bacteriophage endolysin and their applications. *Science Progress*, 99(2): 183 – 199.

Alav, I., Sutton, J. M., Rahman, K. M. 2018. Role of bacterial efflux pumps in biofilm formation. *Antimicrobial Chemotherapy*, 73: 2003 – 2020.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25: 3389 – 3402.

Antimicrobial resistance. 2023. Available: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance#:~:text=Misuse%20and%20overuse%20of%20antimicrobials,be%20resistant%20to%20antimicrobial%20treatment. Accessed: 18 August 2023.

Antoniadou, A., Kantopidou, F., Poulakou, G., Korotzanis, E., Galani, I., Papadamichelakis, E., Kopterides, P., Souli, M. G., Armaganidis, A., Giamarellou, H. 2007. Collistin resistant isolates of *Klebsiella pneumoniae* emerging in intensive care unit patients: First report of multiclonal clusters. *Journal of Antimicrobial Chemotherapy*, 59(4): 786 – 790.

Bagge, N., Hentzer, M., Anderson, J. B., Ciofu, O., Givskov, M., Hoiby, N. 2004. Dynamics and spatial distribution of β-lactamase expression in *Pseudomonas aeruginosa* biofilms. *Antimicrobial Agents and Chemotherapy*, 48: 1168 – 1174.

Bai, J., Yang, E., Chang, P. S., Ryu, S. 2019. Preparation and characterization of endolysin-containing liposomes and evaluation of their antimicrobial activities against gram-negative bacteria. *Enzyme and Microbial Technology*, 128: 40 – 48.

Beciero, A., Liobet, E., Aranda, J., Bengoechea, J. A., Doumith, M., Hornsey, M., Dhanji, H., Chart, H., Bou, G., Livermore, D. M., Woodford, N. 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants *Acinetobacter baumannii* mediated by the *pmrAB* two-component regulatory system. *Antimicrobial Agents and Chemotherapy*, 55(7): 3370 – 3379.

Bertonha, A. F., Silva, C. C. L., Shirakawa, K. T., Trindade, D. M., Dessen, A. 2023. Penicillin-binding protein (PBP) inhibitor development: A 10-year chemical perspective. *Experimental Biology and Medicine*, 29: 1657 – 1670.

Billal, D. J., Feng, J., Leprohon, P., Legore, D., Ouellette, M. 2011. Whole genome analysis of Linezolid resistance in *Streptococcus pneumoniael* reveals resistance and compensatory mutations. *BioMed Central Genomics*, 12(1): 512 – 522.

Black, L. W., Rao, V. B. 2012. Structure, assembly, and DNA packaging of the bacteriophage T4 head. *Advances in Virus Research*, 82: 119 – 153.

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O., Pidddock, L. J. V. 2014. Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology*, 13(1): 42 – 51.

Bon, C. G., Grigg, J. C., Lee, J., Robb, C. S., Caveney, N. A., Eltis, L. D., Strynadka, N. C. J. 2024. Structural and kinetic analysis of the monofunctional *Staphylococcus aureus* PBP1. *Journal of Structural Biology*, 216: 108086.

Borysowski, J., Weber-Dabrowska, B., Gorski, A. 2006. Bacteriophage endolysins as a novel class of antibacterial agents. *Experimental Biology and Medicine*, 231(4): 366 – 377.

Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2): 248 – 254.

Brown, N. M., Goodman, A. L., Horner, C., Jenkins, A., Brown, E. M. 2021. Treatment of methicillin-resistant *Staphylococcus aureus* (MRSA): updated guidelines from the UK. *JAC-Antimicrobial Resistance*, 3(1): p. dlaa114.

Chancey, S. T., Zanher, D., Stephens, D. S. 2012. Acquired inducible antimicrobial resistance in Gram-positive bacteria. *Future Microbiology*, 7(8): 959 – 978.

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., Hanage, W. P. 2015. Antibiotics in agriculture and the risk to human health: how worried should we be? *Evolutionary Applications*, 8(3): 240 – 247.

Ciofu, O., Tolker-Nielsen, T. 2010. Antibiotic tolerance and resistance in biofilms. In: Bjarnsholt, T., Jensen, P., Moser, C., Hoiby, N. (eds) *Biofilm Infections,* 215 – 229.

Cloeckaert, A., Zygmunt, M. S., Doublet, B. 2017. Editorial: Genetics of Acquired Antimicrobial Resistance in Animal and Zoonotic Pathogens, *Frontiers in Microbiology*, 8(2428): 1 – 3.

Coculescu, B. 2009. Antimicrobial changes induced by genetic changes. *Journal of Medicine and Life*, 2(2): 114 – 123.

Date, A. A., Joshi, M. D., Patravale, B. V. 2007. Parasitic diseases: Liposomes and polymeric nanoparticles versus lipid nanoparticles. *Advanced Drug Delivery Reviews*, 59(6): 505 – 521.

Davies, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. *Science*, 264(5157): 375 – 382.

Ding, Y., Zhang, Y., Huong, C., Wong, J., Wong, X. 2020. An endolysin LysSE24 by bacteriophage LPSE1 confers specific bactericidal Activity against multidrug - resistant *Salmonella* strains. *Microorganisms*, 8(5): 737 – 754.

Drlica, K., Zhao, X. 1997. DNA gyrase, Topoisomerase IV, Quinolones. *Microbiology and Molecular Biology Reviews*, 61(3): 377 – 392.

Egido, J. E., Costa, A. R., Aparicio-Maldonado, C., Haas, P-J., Brouns, S. J. J. 2022. Mechanisms and clinical importance of bacteriophage resistance. *FEMS Microbiology Reviews*, 46(1): 1 – 16.

Eloff, J. N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica*, 64: 711 – 713.

Falagas, M. C., Kassiakov, S. K., Saravoloita, L. D. 2005. Collistin: The revival of polymyxins for management of multidrug resistant Gram-negative bacterial infections. *Clinical Infectious Diseases*, 40(9): 1333 – 1341.

Fijin, S. 2016. Antimicrobial effect of probiotics against common pathogens. *Probiotics and Prebiotics in Human Nutrition and Health*, 5772: 191 – 221.

Folgori, L., Bielicki, J., Health, P. T., Sharland, M. 2017. Neonates: Burden of disease and challenges in treatment. *Current Opinion in Infectious Diseases*, 30(3): 281 – 288.

Food Safety. 2022. Available: https://www.who.int/health-topics/foodborne-diseases#tab=tab_1: Accessed: 14 October 2022.

Foster, P. L. 2005. Stress responses and genetic variation in bacteria. *National Institutes of Health*, 569(1-2): 3 – 11.

Fujiwara, A., Fujisawa, M., Hamasaki, R., Kawasaki, T., Fujie, M., Yamada, T. 2011. Biocontrol of *Ralstonia solanacearum* by treatment of with lytic bacteriophages. *Applied and Environmental Microbiology*, 77(12): 4155 – 4162.

Gabrowski, L., Lepek, K., Stasilojc, M., Kosznik-kwasnicka, K., Zdrojewska, K., Maciag-Dorszynska, M., Wegrzyn, G., Wegrzyn, A. 2021. Bacteriophage

encoded enzymes destroying bacterial cell membranes and wall, and their potential use as antimicrobial agent. *Microbiological Research*, 248: 2 – 22.

Ganesh, K., Allam, M., Wolter, N., Bratcher, H. B., Harrison, O. B., Lucidarme, J., Borrow, R., De Gouveia, L., Meiring, S., Birkhead, M., Maiden, M. C. 2017. Molecular characterization of invasive capsule null *Neisseria meningitidis* in South Africa. *BioMed Central*, 17: 1 – 10.

Genin, S., Denny, T. P. 2012. Pathogenomics of the *Ralstonia solanacearum* species complex. *Annual Reviews of Phytopathology*, 50: 67 – 89.

Guo, M., Feng, C., Ren, J., Zhuang, X., Zhang, Y., Zhu, Y., Dong, K., He, P., Guo, X., Qin, J. 2017. A novel antimicrobial endolysin, LysPA26, against *Pseudomonas aeruginosa. Frontiers in Microbiology*, https://doi.org/10.3389/fmicb.2017.00293.

Gutierrez, D., Ruas-madiedo, P., Martinez, B., Rodriguez, A., Garcia, P. 2014. Effective removal of *Staphylococcal* biofilms by the endolysin LysH5. *PLoS ONE*, 9(9): e107307.

Hao, P., Sze, S. K. 2014. Proteomic analysis of protein deamidation. *Current Protocols in Protein Science*, 3:78:24.5: 1 – 14.

Hawkey, P. M. 1998. The origins and molecular basis of antibiotics resistance. *BMJ*, 317(7159): 657 – 660.

Hongbaek, C., Tsuyoshi, V., Bernardt, T. G. 2014. Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. *Cell*, 159(6): 1300 – 1311.

Huan, Y., Kong, Q., Mou, H., Yi, H. 2020. Antimicrobial peptides: Classification, design, application, and research progress in multiple fields. *Frontiers in Microbiology*, doi.org/10.3389/fmicb.2020.582779.

Ikuta, K. S., Swetschinski, L. R., Aquilar, G. R., Sharara, F., Mestrovic, T., Gray, A. P., Weaver, N. D., Wool, E. E., Han, C, Hayoon, A. G., Aali, A., Abate, S. M., Abbasi-Kangevari, M., *et al*. 2022. Global mortality associated with 33 bacterial

pathogens in 2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet*, 400: 2221 – 2248.

Jiang, L., Xiong, Z., Song, Y., Lu, Y., Chen, Y., Schulz, J. S., Li, Y., Liao, J. 2019. Protein-protein affinity determination by quantitative FRET quenching. *Scientific Reports*, 9: 2050.

Jiang, Y., Xu, D., Wang, L., Qu, M., Li, F., Tan, Z., Yao, L. 2021. Characterization of a broad-spectrum endolysin LysSP1 encoded by a *Salmonella* bacteriophage. *Applied Microbiology and Biotechnology*, 105: 5461 – 5470.

Jolivet-Gougeon, A., Bonnaure-Mallet, M. 2014. Biofilms as a mechanism of bacterial resistance. Drug Discovery Today: *Technologies*, 11: 49 – 56.

Kastritis, P. L., Rodrigues, J. P. G. L., M., Folkers, G. E., Boelens, R., Bonvin, A. M. J. J. 2014. Proteins feel more than they see: fine-tuning of binding affinity by properties of the non-interacting surface. *Journal of Molecular Biology*, 426: 2632 – 2652.

Keller, A., Nesvizhskii, A. I., Kolker, E., Aebersold, R. 2002. Empiracle statistical model to estimate the accuracy of peptide identifications made my MS/MS and database search. *Analytical Chemistry*, 74(20): 5383 – 5392.

Kim, H., Lee, H. G., Kwon, I., Seo, J. 2020. Characterization of endolysin LysJH307 with antimicrobial activity against *Streptococcus bovis*. *Animals*, 10(6): 963 – 974.

Kim, S., Lee, D., Jin, J., Kim, J. 2020. Antimicrobial activity of LysSS, a novel phage endolysin, against *Acinetobacter baumannii* and *Pseudomonas aeruginosa. Journal of Global Antimicrobial Resistance*, 22: 32 – 39.

King, A. M., King, D. T., French, S., Brouillette, E., Asli, A., Alexander, J. A. N., Vuvkovic, M., Maiti, S. N., Parr, T. R., Brown, E. D., Malouin, F., Strynadka, N. C. J., Wright, G. D. 2016. Structural and kinetic characterization of diazabicyclooctanes as dual inhibitors of both serine-β-lactamases and penicillin-binding proteins. *ACS Chemical Biology*, 11: 864 – 868.

Kong, M., Ryu, S. 2015. Bacteriophage PBC1 and its and its endolysin as an antimicrobial agent against *Bacillus cereus. Applied and Environmental Microbiology*, 18(7): 2274 – 2283.

Koskella, B., Brockhurst, M. A. 2014. Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS Microbiology Reviews*, 38(5): 916 – 931.

Kumar, V., Ahluwalia, V., Saran, S., Kumar, J., Patel, A. K., Singhania, R. R. 2020. Recent developments in solid-state fermentation for production of microbial secondary metabolites: challenges and solutions. *Bioresource technology*, 323.

Lai, M. J., Lin, N. T., Hu, A., Soo, P. C., Chen, L. K., Chen, L. H., Chang, K. C. Antibacterial activity of *Acinetobacter baumannii phage* ΦAB2 endolysin (LysAB2) against both Gram-positive and Gram-negative bacteria. *Applied Microbiology and Biotechnology*, 90: 529 – 539.

Landers, T. F., Cohen, B., Wittum, T. E., Larson, E. 2012. A review of antibiotic use in food animals: Perspective, policy and potential. *Public Health Reports*, 127(1): 4 – 22.

Lazar, V., Chifiriuc, M. C. 2010. Architecture and physiology of microbial biofilms. *Roumanian Archives of Microbiology and Immunology,* 69(2): 95 – 107.

Lewis, K. 2012. Persister cells: Molecular Mechanisms related to antibiotic tolerance. *Handbook of Experimental Pharmacology*, 211: 121 – 133.

Li, N., Yuan, X., Li, C., Chen, N., Wong, J., Chen, B., Yu, S., Yu, P., Zhong. J., Zeng, H., Wu, S., Yong, X., Yang, M., Zhang, J., Wu, Q., Ding, Y. 2022. *International Journal of Food Microbiology*, 369: 109615.

Li, X., Nikaido, H. 2004. Efflux mediated Drug resistance in bacteria. *Drugs*, 64(2): 159 – 204.

Li, Y., Chen, H., Shu, M., Zhang, C., Bi, Y., Yang, H., Wu, G. 2021. Isolation, characterization, and application of an alkaline resistant virulent bacteriophage JN01 against *Escherichia coli* 0157:H7 in milk and beef. *Food Science and Technology*, 144: 1112666.

Li, Y., Kumar, S., Zhang, L. 2024. Mechanisms of antibiotic resistance and developments in therapeutics strategies to combat *Klebsiella pneumonia* infection. *Infection and Drug Resistance*, 19(17): 1107 – 1119.

Livermore, D. M. 2004. Multiple mechanisms of antimicrobial resistance in *Pseudomonas* spp: our worst nightmare. *Clinical Infectious Diseases*, 35(5): 634 – 640.

Loeffler, J. M., Nelson, D., Fischetti, V. A. 2001. Rapid killing of *Streptococcus pneumoniae* with a bacteriophage cell wall hydrolase. *Science*, 294(5549): 2170 – 2172.

Loessner, M. J. 2005. Bacteriophage endolysins- current state of research and applications. *Current Opinion in Microbiology*, 8(4): 480 – 487.

Lomovskya, O., Warren, M., Lee, A., Galazzo, J., Franko, R., Lee, M., Lee, V. J. 2001. Identification and characterization of inhibitors of multidrug resistant efflux pumps In *Pseudomonas aeruginosa*: Novel agents for combination therapy. *Antimicrobial Agents and Chemotherapy*, 45(1): 105 – 116.

Lopez, D., Vlamakis, H., Kolter, R. 2010. Biofilms. *Cold Spring Harbor Perspectives in Biology*, 2(7): a000398.

Lu, N., Sun, Y., Wang, Q., Qiu, Y., Chen, Z., Wen, Y., Wong, S., Song, Y. 2020. Cloning and characterization of endolysin and holin from *Streptomyces avermitilis* bacteriophage phiSASD1 as potential novel antibiotic candidates. *International Journal of Biological Macromolecules*, 147: 980 – 989.

Ma, F., Xu, S., Tang, Z., Li, Z., Zhang, L. 2021. Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. *Biosafety and Health*, 3(1): 32 – 38.

Macheboeuf, P., Contreras-Martel, C., Job, V., Dideberg, O., Dessen, A. 2006. Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes. *FEMS Microbiology Reviews*, 30: 673 – 671.

Mahamoud, A., Chevalier, J., Alibert-franco, S., Kern, W. V., Pages, J. M. 2007. Antibiotic efflux pumps in Gram-negative bacteria: The inhibitor response strategy. *Journal of Antimicrobial Chemotherapy*, 59(6): 1223 – 1229.

Manyi-Loh, C., Mamphweli, S., Meyer, E., Okah, A. 2018. Antibiotic use in agriculture and its consequential resistance in environmental sources: Potential public health implications. *Molecules*, 23(4): 795 – 843.

Martinez, J. L. 2014. General principles of antibiotic resistance in bacteria. Drug Discovery Today: *Technologies*, 11: 33 – 39.

McDonnell, A., Countryman, A., Laurence, T., Gulliver, S., Drake, T., Edwards, S., Kenny, C., Lamberti, O., Morton, A., Shafira, A., Smith, R., Guzman, J. 2024. Forecasting the fallout from AMR: economic impacts of antimicrobial resistance in humans-a report from the EcoAMR series. Paris (France) and Washington, DC (United States of America): World Organization for Animal Health and World Bank, pp.58. License: CC BY-SA 3.0 IGO.

McManus, M. C. 1997. Mechanisms of bacterial resistance to antimicrobial agents. *American Journal of Health-system Pharmacy,* 54(12): 1420 – 1430.

Medina, E., Pieper, D. H. 2016. Tackling threats and future problems of multidrug resistant bacteria. *Current Topics in Microbiology and Immunology*, 398: 292 – 323.

Moolchandani, K., Sastry, A. S., Deepashree, R., Sistla, S., Harush, B. N., Mandal, J. 2017. Antimicrobial resistance surveillance among intensive care units of a tertiary care hospital in Southern India. *Journal of Clinical and Diagnostic Research*, 11(2): 1 – 7.

Morais, D., Tanoeiro, L., Marques, A. T., Gonçalves, T., Duarte, A., Matos, A. P. A., Vital, J. S., Cruz, M. E. M., Carvalheiro, M. C., Anes, E., Vítor, J. M. 2022. Liposomal delivery of newly identified prophage lysins in a Pseudomonas aeruginosa model. *International Journal of Molecular Sciences*, 23(17): 10143.

Mozafari, R. M., Johnson, C., Hatziantoniou, S., Demetzos, C. 2008. Nanoliposomes and their applications in food nanotechnology. *Journal of Liposome Research*, 18(4): 309 – 327.

Munita, J. M., Arias, C. A. 2016. Mechanisms of Antibiotic Resistance. *Virulence Mechanisms of Bacterial Pathogens*, 5: 481 – 511.

Murray, C. J. L., Ikuta, K. S., Sharara, F., Swetschinshki, L., Aguilar, G. R., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S. C., Browne, A. J., Chipeta, M. G., Fell, F., *et al*. 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet*, 399(10325): 629 – 655.

Nelson, D. C., Schelcher, M., Rodriguez-Rubio, I., Klumpp, J., Pritchard, D. G., Dang, S., Donovan, D. M. 2012. Endolysins as antimicrobials. *Advances in Virus Research*, 83: 299 – 365.

Nelson, D., Loomis, L., Fischetti, V. A. 2001. Prevention and elimination of upper respiratory group A *Streptococci* by using a bacteriophage lytic enzyme. *Proceedings of the National Academy of Sciences*, 98(7): 4107 – 4112.

Nelson, R. 2003. Antibiotic development pipeline runs dry. *The Lancet*, 362(9397): 1726 – 1727.

Nesvizhskii, A. I., Keller, A., Kolker, E., Aebersold, R. 2003. A statistical model for identifying proteins by tandem mass spectrometry. *Analytical Chemistry*, 75(17): 4646 – 4658.

Nikaido, H. 1996 Multidrug efflux pumps of Gram-negative bacteria. *Journal of Bacteriology*, 178(20): 5853 – 5859.

Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews*, 67(4): 593 – 656.

O' Flaherty, S., Coffey, A., Meaney, W., Fitzgerald, G. F., Ross, R. P. 2005. The recombinant phage lysin LysK has a broad spectrum of lytic activity against clinically relevant *Staphylococci*, including methicillin-resistant *Staphylococcus aureus. Journal of Bacteriology*, 187(20): 7161 – 7164.

O'Neill, J. 2016. Tackling drug-resistant infections globally: final report and recommendations. *Review of Antimicrobial Resistance*, London.

Obeso, J. M., Martinez, B., Rodriguez, A., Garcia, P. 2008. Lytic activity of the recombinant *Staphylococcal* Bacteriophage ΦH5 endolysin active against *Staphylococcus aureus* in milk. *International Journal of Food Microbiology*, 128(2): 212 – 218.

Oliveira, H., Thiagarajan, V., Walmagh, M., Silankorva, S., Lavigne, R., Neves-Petersen, M. T., Klustens, L. D., Azeredo, J. 2014. A thermostable *Salmonella* phage endolysin, Lys68, with broad bactericidal properties against Gram-negative pathogens in presence of weak acids. *PLoS One* 9(12): 1 – 11.

Palzkill, T. 2012. Metallo-β-lactamase structure and function. Annals of the New York *Academy of Science*, 1277(1): 91 – 104.

Park, B. H., Levy, S. B. 1988. The cryptic Tetracycline resistance determinant on Tn4400 mediates Tetracycline degradation as well as Tetracycline efflux. *Antimicrobial Agents and Chemotherapy*, 32(12): 1797 – 1800.

Paulsen, I. T., Park, J. H., Choi, P. S., Saier, M. H. 1997. A family of Gram-negative bacterial outer membranes factors that function in the export of proteins, carbohydrates, drugs and heavy metal Gram-negative bacteria. *FEMS Microbiology Letters*, 156(1): 1 – 8.

Peacock, J. S., Paterson, G. K. 2015. Mechanisms of Methicillin resistance in *Staphylococcus aureus*. *Annual Reviews of Biochemistry*, 84: 577 – 601.

Portilla, S., Fernandez, L., Gutierrez, D., Rodriguez, A., Garcia, P. 2020. Encapsulation of the antistaphylococcal endolysin LysRODI in Ph-sensitive liposomes. *Antibiotics*, 9(5): 242 – 250.

Principi, N., Silvestri, E., Esposito, S. 2019. Advantages and limitation of bacteriophages for the treatments of bacterial infections. *Frontiers in Pharmacology*, 8(10): 513.

Reygaert, W. C. 2018. An overview of the antimicrobial resistance mechanisms of bacteria. *Aims Microbiology*, 4(3): 482 – 501.

Rudd, K. E., Johnson, S. C., Agesa, K. M., Shackelford, K. A., Tsoi, D., Kievlan, D. R., Colombara, D. V., Ikuta, K. S., Kissoon, N., Finfer, S., Fleischmann-Struzek, C., Machado, F. R., Reinhart, K. K., Rowan, K., *et al*. 2020. Global, regional,

and national sepsis incidence and mortality, 1990-2017: Analysis for the Global Burden of Disease Study. *Lancet*, 395: 200 – 211.

Salahshoori, I., Golriz, M., Nobre, M. A. L., Mahdavi, S., Malekshah, R. E., Javdani-Mallak, A., Jorabchi, M. N., Khonakdar, H. A., Wang, Q., Mohammadi, A. H., Mirnezami, S. M. S., Kargaran, F. 2024. Simulation-based approaches for drug delivery systems: Navigating advancements, opportunities, and challenges. *Journal of Molecular Liquids*, 395: 123888.

São-José, C. 2018. Engineering of Phage-derived lytic enzymes: Improving their potential as antimicrobials. *Antibiotics*, 7(2): 29 – 60.

Savva, G. G., Dowy, J. S., Moussa, K. N., Halzenburg, A., Young, R. Y. 2014. Stable micron scale holes are a general feature of canonical holins. *Molecular Microbiology*, 91(1): 57 – 65.

Schmelcher, M., Donovan, D. M., Loessner, J. M. 2012. Bacteriophage endolysins as novel antimicrobials. *Future Microbiology*, 7(10): 1147 – 1171.

Serra-Burriel, M., Keys, M., Campillo-Artero, C., Agodi, A., Barchitta, M., Gikas, A., Palos, C., Lopez-Casasnovas, G. 2020. Impact of multi-drug-resistant bacteria on economic and clinical outcomes of healthcare associated infection in adults: Systematic review and meta-analysis. *PLoS One*, 15(1): 1 – 14.

Shilabin, A. G., Dzhekieva, L., Misra, P., Jayaram, B., Pratt, R. F. 2012. 4-Quinolones as noncovalent inhibitors of high molecular mass penicillin-binding proteins. *ACS Medical Chemistry Letters*, 3: 592 – 595.

Son, B., Kong, M., Lee, Y., Ryu, S. 2021. Development of a novel chimeric endolysin, Lys109 with enhanced lytic activity against *Staphylococcus aureus*. *Frontiers in Microbiology*, 11: 615887.

Tahlan, K., Jensen, S. E. 2013. Origins of the Beta-lactam rings in natural products. *The Journal of Antibiotics*, 66: 401 – 410.

Taylor, T. M., Weiss, J., Davidson, P. M., Bruce, B. D. 2007. Liposomal nano capsules in food science and agriculture. *Critical Reviews in Food Science and Nutrition*, 45(7-8): 587 – 605.

Teixeira, M. L., Santos, J. D., Silveira, N. P., Brandelli, A. B. 2008. Phospholipid nanovesicles containing a bacteriocin-like substance for control of *Listeria monocytogenes*. *Innovative Food Science & Emerging Technologies*, 9(1): 49 – 53.

Tovchigrechko, A., Vakser, I. A. 2006. GRAMM-X public web server for protein-protein docking. *Nucleic Acid Research*, 34: W310 - W314.

Vaara, M. 1993. Antibiotic super susceptible mutants of *Escherichia coli* and *Salmonella typhimurium*. *Antimicrobial Agents and Chemotherapy*, 37(11): 2255 – 2260.

Vangone, A., Bonvin, A. M. J. J. 2015. Contact-based prediction of binding affinity in protein-protein complexes. *eLife*, 4: e07454.

Vetting, M. W., Hegde, S. S., Wang, M., Jacoby, G. A., Hooper, D. C., Blanchard, J. S. 2011. Structure of QnrB: A plasmid mediated Fluroquinalone resistance factor. *Journal of Biological Chemistry*, 286(28): 25265 – 25273.

Viertal, T. M., Ritter, K., Horz, H. P. 2014. Viruses' vs bacteria – novel approaches to phage therapy as a tool against multidrug-resistant pathogens. *Journal of Antimicrobial Chemotherapy*, 69(9): 2326 – 2336.

Wallace, A. C., Laskowski, R. A., Thornton, J. M. 1996. LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Engineering*, 8: 127 – 134.

Webber, M. A. 2003. The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy*, 51(1): 9 – 11.

WHO Bacterial Priority Pathogens List, 2024: Bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. Geneva: World Health Organization. License: CC BY-NC-SA 3.0 IGO.

Wright, G. D. 2005. Bacterial resistance to antibiotics: Enzymatic degradation and modification. *Advanced Drug Delivery Reviews*, 57(10): 1451 – 1470.

Young, R. 1992. Bacteriophage lysis: Mechanism and regulation. *Microbiological Reviews*, 56(3): 430 – 481.

Young, R. Y., Wong, I., Roof, W. D. 2000. Phages will out: Strategies of host cell lysis. *Trends in Microbiology*, 8(3): 120 – 128.

Zapun, A., Contreras-Martel, C., Vernet, T. 2008. Penicillin-binding proteins and β-lactam resistance. *FEMS Microbiology Reviews*, 32: 361 – 385.

Zdorovenko, E. L., Besarab, N. V., Shashkov, A. S., Novic, G. I., Shirokov, A. A., Burov, A. M., Knirel, X. A. 2018. Investigation of O-polysacharides from bacterial strains of *Pseudomonas* genus as potential receptors of bacteriophages BIN-45. *International Journal of Biological Macromolecules*, 118: 1065 – 1072.

Zhang, H. 2016. Thin-film hydration followed by extrusion method for liposome preparation. *Liposomes*, 1522: 17 – 22.

Zhang, K., Li, X., Wang, Z., Li, G., Ma, B., Chen, H., Li, N., Yang, H., Wang, Y., Liu, B. 2021. Systemic expression, purification, and initial structural characterization of bacteriophage T4 proteins without known structure homologs. *Frontiers in Microbiology*, 12: 674415.