Studies on Use of Bacteriophages in Treating Antibiotic-Resistant Infections of Diabetic Foot Ulcers

by

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Thesis

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Abbreviations

µg	Microgram
µl	Microliter
ATCC	American type culture collection
Bap	Biofilm associated protein
BGL	Blood glucose level
BSA	Bovine serum albumin
CFU	Colony forming unit
cGMP	Current good manufacturing product
CRISPR-cas	Clustered regularly interspaced short palindromic repeats associated system
CV	Crystal violate
DFU	Diabetic foot ulcer
DNA	Deoxyribonucleic acid
EPS	Extracellular polysaccharide substance
GFP	Green fluorescence protein
HCCA	Alpha-cyano-4-hydroxy cinnamic acid
HQNO	2-heptyl-4-hydroxyquinoline N-oxide
ICTV	International committee for taxonomy of viruses
IP	Intraperitoneal
IU	International unit
L	Littre
LB	Luria-Bertani
m/z	Mass charge ratio
MALDI-TOF MS	Matrix assisted laser desorption/ionization time of flight mass spectrometry
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
ml	milliliter
mM	Millimolar
mmol	Millimole
MOI	Multiplicity of infection
MRSA	Methicillin resistant *S. aureus*

MSA	Mannitol salt agar
MSSA	Methicillin susceptible *S. aureus*
NCCLS	National committee for clinical laboratory standards
^{o}C	Degree centigrade
OD	Optical density
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
PFU	Plaque forming unit
PVL	Panton-Valentine leucocidin
RFU	Relative fluorescence unit
RM	Restriction-modification
RNA	Ribonucleic acid
rpm	revolutions per minute
S. aureus	*Staphylococcus aureus*
SCV	Small colony variant
Sie	Super-infection exclusion
STZ	Streptozotocin
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TTC	Tetracycline

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

________________________ June 2020

Legesse Garedew Kifelew Date

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Dedication

To my wife Alemwork Abate Kebede, who teach me selfless sacrifice,

and

To my children, Brook Legesse Garedew and Nahom Legesse Garedew, who give meaning to everything I do.

You have made me more fulfilled than I could have ever imagined.

Summary

This work is in response to two severe problems confronting global health systems: diabetes and antibiotic resistance. Diabetic foot ulcers (DFUs) are open lesions that fail to heal in the foot of a diabetic patient. About one in four people living with diabetes develop diabetic foot ulceration during their lifetime. DFUs causes close to 90% of limb amputations among persons with diabetes. The 5-year mortality rate following foot amputation due to DFUs is up to 74%. Most DFUs become infected, and antibiotic-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* are often isolated from DFUs. The increasing failure of antibiotics prompts the development of bacteriophages ("phages") as alternative anti-infective agents.

An important aspect of bringing phages to the clinic is that they are made to high standards. We have access to phage cocktails AB-SA01, against *S. aureus*, and AB-PA01, against *P. aeruginosa*, both of which are made to current good manufacturing practice (cGMP) standard. This study sought to investigate the lytic efficacy of AB-SA01 and its three component phages against *S. aureus*, and AB-PA01 and its four component phages against *P. aeruginosa* clinical isolates. Because wound infections are often polymicrobial and contain bacteria in biofilm form, this work sought to examine the efficacy of phages in such a setting.

Findings in chapters 2 and 3 indicate that phage cocktails AB-SA01 and AB-PA01 and their components are strongly lytic on most clinical isolates in planktonic and biofilms states. Moreover, these phage cocktails produced significant biomass reduction in single-species biofilms similar or superior to antibiotics used as a positive control. The efficacy and host range of phage cocktails AB-SA01 and AB-PA01 and their components suggest the

enormous potential of phages in the treatment of antibiotic-resistant S. *aureus* and *P. aeruginosa* infections.

Real-time monitoring using spectrophotometry, supported by the colony count method, demonstrates that the fluorescence and population density of mCherry *S. aureus* KUB7 and GFP *P. aeruginosa* PAO1 significantly decreased when treated by their phage cocktail alone and the mixture of the two phage cocktails, similar to gentamicin treatment, as detailed in chapter 4. The findings in chapter 4 show that AB-SA01 and AB-PA01 effectively lysed their hosts in the presence of non-susceptible bacteria, both in planktonic and biofilm states. Moreover, the findings demonstrate that mCherry- and GFP-based mixed-species microplate assay using spectrophotometry combines reproducibility, rapidity, ease of management, and compatibility with high-throughput screening.

The results of multidrug-resistant *S. aureus* infected diabetic mice wound treatment show that AB-SA01 effectively decreased the bacterial load and significantly improved wound healing, similar to vancomycin treatment. In contrast, wounds of saline-treated mice showed no healing, but expanded and became inflamed, ulcerated, and suppurating. No adverse effect related to the application of the phage cocktail was observed. Hence, the results suggest that topical phage cocktail treatment may be useful in treating antibiotic-resistant *S. aureus* infections.

Overall, this thesis shows that the lytic efficacy, broad host range, and significant biofilm biomass reduction capability, in single-species and mixed-species biofilms, of the phages, makes them suitable candidates for therapeutic use in the DFU setting. Further investigation is required to evaluate the clinical application and off-target effect of phages and phage resistance development.

Thesis structure

This thesis is structured in manuscript format, with chapters 2 to 5 submitted or under preparation for submission on peer-reviewed journals. Chapters 4 and 5 are published and in press in Viruses and BMC Microbiology journals, respectively. Chapters 2 and 3 are under preparation to be submitted to Biofouling and Frontiers in Microbiology journals, respectively. Because the studies are continuations of others or related in many ways, redundancies may present mainly in the introductions and methodology sections. Chapter 2 describes the efficacy and host range of AB-SA01 and its component phages on laboratory strains and clinical isolates of diabetic foot ulcers on *in vitro* planktonic and biofilm states. Chapter 3 analyzes the lytic effect and host range of AB-PA01 and its components on laboratory strains and isolates collected from diabatic foot ulcer patients on *in vitro* planktonic and biofilm phases. Chapter 4 studies the lytic efficacy of phage cocktails AB-SA01 and AB-PA01 on their hosts in mixed-species planktonic and biofilm cultures. Chapter 5 examines the effect of AB-SA01 on diabetic mice wound infection caused by multidrug-resistant (MDR) *S. aureus*. Chapter 6 discusses the general findings of chapters 2 to 5. All cited literature is provided in one collated reference list at the end of this thesis to avoid redundancy. Although the nominative form "we" is used in the manuscripts under preparation or submitted for journals, manuscripts of Chapters 2 - 5, the work presented was undertaken by myself under the supervision of the coauthors.

Chapter 1

General Introduction

Chapter 1: General introduction

1. Introduction

1.1 Diabetic foot ulcer infection

Diabetic foot ulcer (DFU) is defined as foot, in a diabetic patient, with an open sore caused by a break in the skin that fails to heal. It is a devastating complication of diabetes often associated with neuropathy and peripheral arterial disease of lower limbs (Alexiadou and Doupis, 2012), as illustrated in Figure 1.1. About one in four people living with diabetes develop diabetic foot ulceration during their lifetime (Armstrong *et al.*, 2017). DFU is one of the most common reasons for diabetic-related lower extremity amputation (Uçkay *et al.*, 2015). Over 50% of DFU become clinically infected (Lavery *et al.*, 2007). DFU infections are costly complications of the diabetic foot associated with high morbidity, mortality, treatment costs, risk of lower extremity amputation, and poor quality of life (Alexiadou and Doupis, 2012).

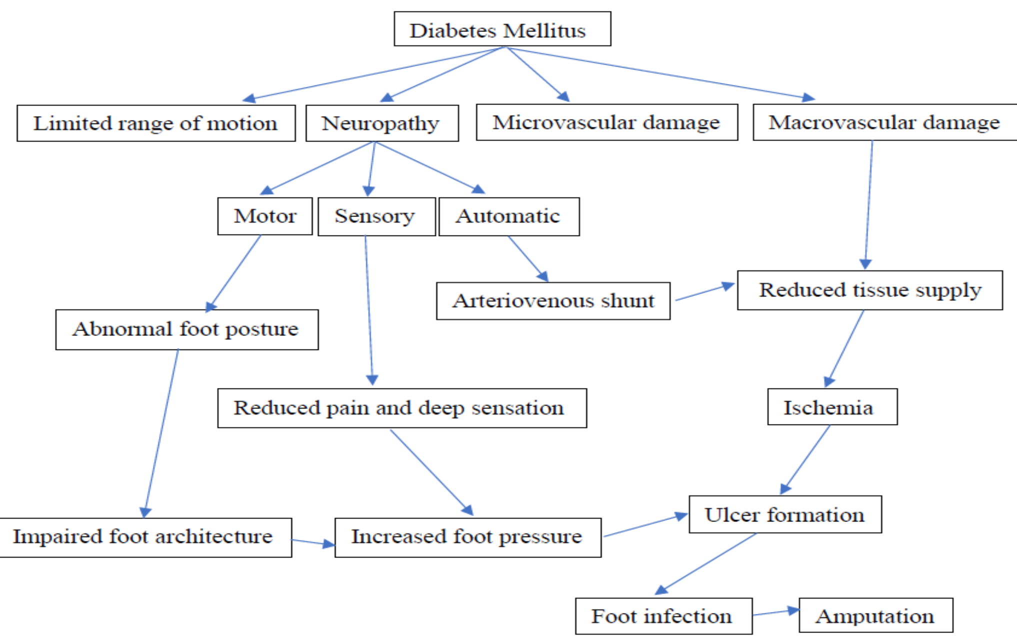

Figure 1. 1 Pathogenesis of DFU infection (adapted from (Turan et al., 2015)

DFU infections are usually polymicrobial, mainly bacterial agents such as *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Lee *et al.*, 2017; Malik *et al.*, 2013; Ramakant *et al.*, 2011). *S. aureus* and *P. aeruginosa* are the most common bacterial species isolated from DFUs, with up to 93.5% and 52.2% detection rate, respectively (Gjødsbøl *et al.*, 2006). They are frequently isolated together from chronic wound infections (Bessa *et al.*, 2015; Trivedi *et al.*, 2014; Körber *et al.*, 2010). Limited studies showed that *S. aureus-P. aeruginosa* mixed-species infections are more virulent than single-species infections (Pastar *et al.*, 2013; Hendricks *et al.*, 2001). Because of the formation of polymicrobial biofilms, expression of virulence factors, and synergistic interactions between *P. aeruginosa* and *S. aureus*, delayed wound healing is seen compared to single species infected wounds (Pastar *et al.*, 2013).

Due to the impaired immune defense around the necrotic tissue of DFU, low-virulence bacteria can also play a pathogenic role (Lipsky, 1999). Close to 70% - 85% of lower-limb amputations in patients with diabetes are caused by biofilm-related foot ulcer infections (Malik *et al.*, 2013; Dowd *et al.*, 2008). Bacteria residing within biofilms are resistant to many antibiotics (Uçkay *et al.*, 2016; Malik *et al.*, 2013; Ramakant *et al.*, 2011; Davis *et al.*, 2006). *P. aeruginosa* and *S. aureus* usually form biofilms *in vivo* that could contribute to their antibiotic resistance characteristic (Ramakant *et al.*, 2011; Dowd *et al.*, 2008). Difficulty in growing different bacterial species together *in vitro* has made the study of species interactions challenging; this is true for *S. aureus* and *P. aeruginosa* (Kahl, 2017; Armbruster *et al.*, 2016; Korgaonkar *et al.*, 2013; Mashburn *et al.*, 2005). Though they are commonly isolated together from mixed-species infections, reports indicate that *P. aeruginosa* often kills *S. aureus* both *in vitro* (Kumar and Ting, 2015; DeLeon *et al.*, 2014) and *in vivo* (Filkins *et al.*, 2015; Pernet *et al.*, 2014).

1.2 *S. aureus*

S. aureus is a non-motile and facultative anaerobic bacterium with 0.5 - 1.5 μm diameter. It is a Gram-positive coccus that typically grows in clusters; hence the name from Greek word *staphylé* to say, "a bunch of grapes" and *kokkos* means "berry" or "seed" (Wilson, 1987) and *aureus* from Latin word *aurum* meaning "gold" to describe its golden color colonies (Stryjewski and Corey, 2014), as shown in Figures 1.2A and 1.2B. *S. aureus* can grow at high salt concentration and temperature ranging from 18 ^{o}C to 40 ^{o}C (Tong *et al.*, 2015). It is also strongly resistant to hostile environmental conditions, including low water activity and high osmotic pressure (Gnanamani *et al.*, 2017). Most *S. aureus* strains express a polysaccharide capsule that protects the bacterium from phagocytosis (Vandenesch *et al.*, 1994).

Figure 1. 2 Characteristics of *S. aureus* colonies and cells: A - golden colonies of *S. aureus* on nutrient agar, B - yellow colonies of *S. aureus* on mannitol salt agar, and C - cluster formation of *S. aureus* in Gram-staining. (Adopted from (Murray *et al.*, 2015)).

S. aureus produces several toxins that contribute to its virulence including cytolytic toxins α, β, δ, γ, and Panton-Valentine leucocidin (PVL), and exfoliative toxins such as enterotoxins and toxic shock syndrome toxin-1 (Prevost *et al.*, 1995). It also contains enzymes that help its spread within the human body and manipulate immune responses. Some of the enzymes are (i) coagulase - converts fibrinogen to fibrin, (ii) catalase - converts hydrogen peroxide to water and oxygen, (iii) hyaluronidase - breaks down hyaluronic acid, and (iv) staphylokinase - inactivates the bactericidal α-defensins (Dunyach-Remy *et al.*, 2016; Dinges *et al.*, 2000).

These virulence factors help *S. aureus* to establish infection through evading host immune responses and facilitating tissue attachment and invasion (Gnanamani *et al.*, 2017).

S. aureus occurs as a human commensal, a common colonizer of the skin, anterior nares, oropharynx, gastrointestinal tract, and urogenital tract (Messad *et al.*, 2015). Approximately 30% and 70% of the human population carry *S. aureus* in their anterior nares permanently and periodically, respectively (Mulcahy and McLoughlin, 2016; Wertheim *et al.*, 2005; Kluytmans *et al.*, 1997). Wound infections, pneumonia, osteomyelitis, meningitis, endocarditis, and thrombophlebitis are some of the diseases caused by *S. aureus*. Moreover, it is one of the most prevalent causes of nosocomial infections (Tong *et al.*, 2015; Lowy, 1998).

S. aureus is one of the most commonly isolated bacteria from DFUs, including from soft tissue infections and osteomyelitis. Its toxins and virulence factors have an essential role in deepening and spreading the DFU infections through considerable proteolytic and toxic activity (Viquez-Molina *et al.*, 2018). Of all *S. aureus* infections, about 15% were caused by methicillin-resistant *S. aureus* (MRSA) strains. The prevalence of MRSA in DFUs is estimated at 30% (Lavery *et al.*, 2014). The high prevalence of *S. aureus* and the rapid rise of its antibiotic resistance forced the development of guidelines recommending empirical antibiotic therapy with anti-Staphylococcal coverage for all patients with DFU infections (Lipsky *et al.*, 2016). MRSA nasal colonization and infection with *S. aureus* strains resistant to various antibiotics are risk factors for higher MRSA infection prevalence in DFU patients (Lavery *et al.*, 2014).

1.3 *P. aeruginosa*

P. aeruginosa belongs to the genus Pseudomonas (from the Greek words "*pseudes*" and "*monas*" meaning false unit). It is a rod-shaped with 0.5 to 0.8 μm width, and 1.5 to 3.0 μm

lengths. *P. aeruginosa* is a Gram-negative, facultative aerobic, and motile bacteria (Sneath *et al.*, 1986). *P. aeruginosa* grows best at 37 °C (Anzai *et al.*, 2000). Its colonies appear mucoid because most strains produce a thick and viscous polysaccharide capsid (Stehling *et al.*, 2008), as shown in Figure 1.3. Most strains of *P. aeruginosa* produce fluorescent pigments, including pyocyanin (blue), pyorubin (red-brown), and pyoverdine (yellow-green). These pigments interfere with host cell respiration (pyocyanin) and iron transport (pyoverdine) and protect the bacteria from hydrogen peroxidase (pyorubin) (Orlandi *et al.*, 2015; Jayaseelan *et al.*, 2014; Rodríguez-Rojas *et al.*, 2009; Visca *et al.*, 2007; Lau *et al.*, 2004).

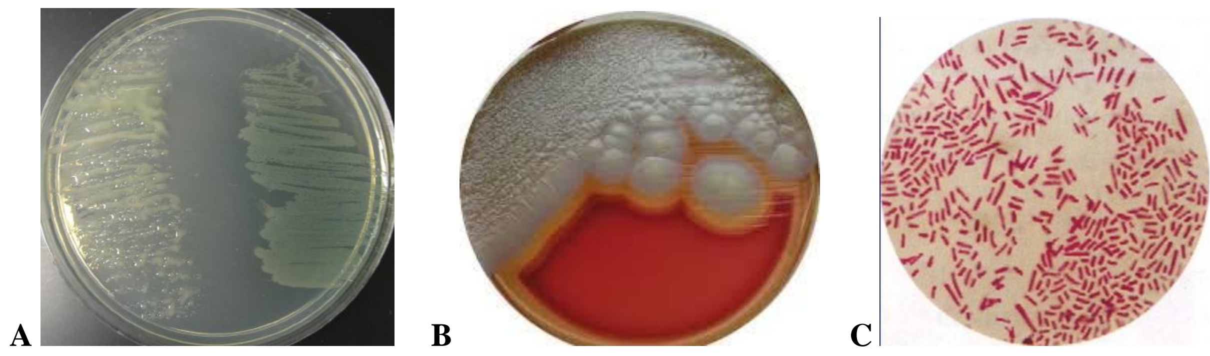

Figure 1. 3 Characteristics of *P. aeruginosa* colonies and cells: **A** - colonies of *P. aeruginosa* on nutrient agar, B - metallic sheen colonies of *P. aeruginosa* on Mac Conkey agar, and **C -** road-shaped *P. aeruginosa* in Gram-staining. (From (Murray *et al.*, 2015)).

P. aeruginosa is armed with several virulence factors, including toxins, enzymes, adhesins, and pigments that collectively help it to escape the immune system and propagate. Among the toxins, exotoxin A contributes to its pathogenesis (Jaffar-Bandjee *et al.*, 1995). Once exotoxin A is inside the host cell, it can disrupt host cell protein synthesis, resulting in tissue damage (Breidenstein *et al.*, 2011; Lister *et al.*, 2009; Hancock, 1998). Its regulatory mechanisms and virulence factors render *P. aeruginosa* metabolically versatile and capable of inhabiting humans, animals, plants, and environments (Breidenstein *et al.*, 2011; Frimmersdorf *et al.*, 2010).

P. aeruginosa is resistant to many commonly used antibiotics, weak antiseptics, and high concentrations of salts and dyes. It shows high intrinsic resistance to a wide variety of antibiotics, including β-lactams, fluoroquinolones, and aminoglycosides, mainly because of its membrane low permeability due to the absence of efficient transmembrane proteins (porins) that form water-filled pores in the hydrophobic part of the membrane (Nicas and Hancock, 1983). This characteristic facilitates increased efflux and enzymatic modification of antibiotics (Hancock, 1998; Nicas and Hancock, 1983). Moreover, *P. aeruginosa* can develop acquired antimicrobial resistance through horizontal genetic elements transfer and mutation. Conjugation, transformation, and transduction can transfer resistance islands, prophages, plasmids, integrons, and transposons that harbor antibiotic resistance genes to the next generation (Breidenstein *et al.*, 2011). The transfer of antibiotic-resistant genes may exacerbate the antimicrobial-resistance characteristics of the bacteria (Breidenstein *et al.*, 2011; Li *et al.*, 2000).

P. aeruginosa is a predominant pathogen causing severe diseases in immunocompromised individuals (Høgsberg *et al.*, 2011). *P. aeruginosa* is one of the causes of treatment failure due to its capability of rapidly acquiring resistance to a wide range of antibiotics (Serra *et al.*, 2015; Lister *et al.*, 2009). Antibiotic resistance of *P. aeruginosa* isolates has been significantly increased during the past two decades (Chatterjee *et al.*, 2016; Breidenstein *et al.*, 2011). *P. aeruginosa* infected wound is characterized by a significantly broader ulcer area coverage and a delayed or poor healing process (Gjødsbøl *et al.*, 2006). *P. aeruginosa* is among the most commonly isolated bacteria from DFU and often localized in the deepest region of the wound, usually as a biofilm (Fazli *et al.*, 2009).

1.4 Bacterial biofilms

Studies using advanced scanning techniques have demonstrated the micro-heterogeneity of biofilms with variable distribution of cells, matrix, and fluid-filled channels and pores (Wood *et al.*, 2000). Biofilm formation will begin by adherence to surfaces with the assistance of flagella and pili in Gram-negative (Bjarnsholt *et al.*, 2013) or surface proteins in Gram-positive bacteria (Cucarella *et al.*, 2001). Some bacterial species capable of producing proteins like biofilm-associated protein (*bap*) are found to be surface adherent and strong biofilm producers. *Bap* also involves in pathogenesis, causing a persistent infection (Cucarella *et al.*, 2001). Cell-to-cell signaling is required for biofilm formation (Macia *et al.*, 2014) and may depend on the concentration of diffusible signal molecules such as second messengers and small ribonucleic acids (RNAs) (Bjarnsholt *et al.*, 2013). Various micro-organisms share a common milieu and coexist in niches forming multispecies biofilms (Thein *et al.*, 2007). The synergistic interactions of bacterial species may contribute to the delay in healing from infection and antibiotic tolerance by co-infecting bacteria (Dalton *et al.*, 2011).

Biofilm formation occurs in response to environmental changes that induce multiple regulatory mechanisms, which ultimately lead to spatial and temporal reorganization of the bacterial cells as a means of survival (O'Toole *et al.*, 2000). This phenomenon affects the expression of surface molecules and virulence factors and nutrient utilization of the bacteria (Kostakioti *et al.*, 2013). Biofilm can be formed on biotic and abiotic surfaces (O'Toole and Wong, 2016). Bacterial biofilms consist of aerobic and anaerobic growing subpopulations (Bjarnsholt *et al.*, 2013). Bacteria cells occupy 5% - 30% of the biofilm volume (Macia *et al.*, 2014). The remaining portion of the biofilm is EPS, composed mainly of exopolysaccharides, and small amounts of protein, DNA, bacterial lytic products, and compounds from the host. The nature, concentration, and arrangement of these components determine the density and

porosity of the biofilm (Flemming and Wingender, 2010). The EPS has the potential to modify the response to antimicrobial treatments through its action as a diffusion barrier and neutralizer (Gilbert *et al.*, 2002).

Bacteria residing in biofilms are more resistant to the chemical and physical effects of the environment than their planktonic counterparts (Steenackers *et al.*, 2016; Gilbert *et al.*, 2002). Bacterial cells present in biofilms are thought to be up to 1000 times more resistant to antibiotics and biocides than their planktonic cells (Høiby *et al.*, 2010; Ceri *et al.*, 1999). An estimated 65% - 80% of all human infections, especially chronic wounds such as DFU, are biofilm-related (Percival *et al.*, 2012; Coenye and Nelis, 2010). Biofilms are tolerant to physical and chemical effects due to the complex nature of their extracellular matrix and the presence of metabolically inactive persister cells (Harper *et al.*, 2014). Thus, the need for effective biofilm elimination strategies becomes imperative (Kostakioti *et al.*, 2013).

1.5 Treatment of DFU

The main aim of DFU management is to achieve wound healing and wound closure. The complete treatment of DFU includes the draining of invasive infections, debriding necrotic tissues, administering appropriate antibiotics, and performing vascular reconstruction (Bradbury *et al.*, 2005). The wound healing process is characterized by homeostasis, inflammation, and proliferation and remodelling of keratinocytes, fibroblasts, Langerhans cells, and endothelial cells (Wang *et al.*, 2012). One of the challenges in the treatment of DFU is co-infection by antibiotic-resistant bacteria such as *P. aeruginosa* and *S. aureus* (Fazli *et al.*, 2009; Gjødsbøl *et al.*, 2006). Often, *S. aureus* is localized in the top while *P. aeruginosa* is in the deepest and broader area of wounds (Serra *et al.*, 2015; DeLeon *et al.*, 2014; Gjødsbøl *et al.*, 2006). Studies showed that the antibiotic resistance of *P. aeruginosa*

and *S. aureus* increase when they grow together (DeLeon *et al.*, 2014) and exist as co-infection in wound ulcers that deter healing processes (Pastar *et al.*, 2013).

Biofilms mostly contain polymicrobial population adherent to surfaces using extracellular polymeric materials that have antiphagocytic properties. The extracellular polymeric material can inactivate the complement system and antibodies to cause tissue damage and chronic inflammation (Wolcott *et al.*, 2013). Hyperglycaemia enhances oxidative stress and increased interleukin-8 secretion from keratinocytes that may contribute to the delayed healing of diabetic wounds (Lan *et al.*, 2013). Removing biofilm through debridement supported by antibiotics could improve wound healing (Malik *et al.*, 2013). Vancomycin plus either ciprofloxacin, meropenem, ertapenem, or piperacillin/tazobactam are antibiotics recommended through systemic route for *S. aureus* and *P. aeruginosa* co-infection (Lipsky *et al.*, 2012b). Except for the experimental use of moxifloxacin (Bessa *et al.*, 2015), no topical broad-spectrum antibiotic is available to treat DFU to date. Furthermore, no drug is designed against bacteria in biofilms.

1.6 Animal models for DFU infection and treatment

In vitro and *in vivo* validations are required before the application of therapeutic agents in clinical settings. As *in vitro* experiments fail to reproduce the complex physiology of the host organism and pathophysiology of the infected wound, the *in vivo* models play an important role in ensuring clinical relevance (Mendes *et al.*, 2012a). When the *in vitro* tests are showing promising findings, the next logical step is to study the agent in an animal model. Wound infection and treatment effect studies have been carried out in animal models, including mice (Turner *et al.*, 2017; de Mayo *et al.*, 2017), rats (Mendes *et al.*, 2012a), rabbits (Wills *et al.*, 2005), and pigs (Velander *et al.*, 2008). Different wound infection and treatment models, including incisional and excisional wounds in animals rendered diabetic through chemical

induction or genetic modification, are also documented (Turner *et al.*, 2017; Abendroth *et al.*, 2010; Galiano *et al.*, 2004).

Genetically diabetic animal models are limited in availability and expensive. Besides, they are often obese that may impede wound closure through physiological dysfunction and excess subcutaneous fat accumulation (Abedon, 2012; Chen *et al.*, 2011; Davidson, 1998). Chemically induced diabetes model is recommended when testing therapeutic agents (Davidson, 1998); hence it is employed in this study. Injection of streptozotocin (STZ) is widely in use to induce diabetes as it is cost-effective, rapid, and known to induce type 2 diabetes mellitus in most species and strains of rodents (Deeds *et al.*, 2011). STZ (N-nitroso derivative of glucosamine) is a broad-spectrum antibiotic extracted from *Streptomyces acromogenes* bacteria (Hayashi *et al.*, 2006). STZ is used for the treatment of the human pancreas islet cell metastatic carcinoma (Deeds *et al.*, 2011; Hayashi *et al.*, 2006). It induces rapid and irreversible necrosis to insulin-producing β-cells of rodents' pancreatic islets (Lenzen, 2008). STZ is taken up through GLUT2 glucose transporter of the cell membrane in rodents and causes alkylation of DNA and β-cell death (Lenzen, 2008; Szkudelski, 2001). Sensitivity to STZ is highly variable in rodent species and strains (Deeds *et al.*, 2011; Hayashi *et al.*, 2006). Rodents also show gender difference to STZ toxic effect; males are profoundly affected by STZ toxicity (Cortright *et al.*, 1996; Leiter, 1985).

An excisional wound model in mice has been used to evaluate the efficacy of topically applied antibacterial agents (Davidson *et al.*, 2013). Mouse wound models are commonly used because of low cost and ease for management (Park *et al.*, 2014). Unlike the re-epithelization and the granulation process of human wound healing, mouse wound healing is through skin constriction (Park *et al.*, 2014; Dunn *et al.*, 2013). A splint excisional wound model using a silicone sheet that prevents excessive skin contraction is used to mimic human

wound healing type in mice (Park *et al.*, 2014; Dunn *et al.*, 2013; Lindblad, 2008). Treatment in all these methods is by antibiotic agents. In this thesis, the use of bacteriophage treatment is studied.

1.7 Bacteriophages

Bacteriophages ("phages") are viruses that infect bacteria. The word "phage" derives from the Greek word "phagein" meaning, "to eat"; hence bacteriophages mean "bacteria eaters". Phages were first discovered independently by the British scientist Frederick William Twort (Twort, 1915) and the French-Canadian scientist Felix Hubert d'Herelle (d'Herelle, 1917) in 1915. However, d'Hérelle was the first scientist who studied phages to combat bacterial infections. d'Hérelle is also the author of the terms bacteriophages and plaque (the small clear areas seen in agar plates caused by bacterial killing by phages) (d'Hérelle and Smith, 1922; d'Herelle, 1917).

Commercialization of phages was started in the 1920s by large companies of the time (Pirnay *et al.*, 2011; Carlton, 1999). Following the discovery of penicillin in 1928 by Alexander Fleming, the treatment of infectious diseases relied almost exclusively on antibiotics. Since then, phage therapy was largely forgotten until recently in most parts of the globe except in the former Soviet Union and Eastern Europe (Chanishvili *et al.*, 2002; Alisky *et al.*, 1998). Phages are 20 - 200 nm in size (Ly-Chatain, 2014). To date, the International Committee for Taxonomy of Viruses (ICTV) recognizes 13 phage families, as listed in Table 1.1.

Table 1. 1 ICTV classification of phages (adapted from (Ackermann and Prangishvili, 2012)).

Family	**Morphology**	**Nucleic acid**	**Examples**
Myoviridae	Non-enveloped, contractile tail	Linear dsDNA	Tailed phage, T4
Siphoviridae	Non-enveloped, non-contractile tail	Linear dsDNA	Phage λ
Podoviridae	Non-enveloped, non-contractile tail	Linear dsDNA	Coliphage T7
Tectiviridae	Non-enveloped, isometric	Linear dsDNA	Phage PRDI
Corticoviridae	Non-enveloped, isometric	Circular dsDNA	PM2
Lipothrixviridae	Enveloped, rod-shaped	Linear dsDNA	Thermoproteus Φ phage I
Plasmaviridae	Enveloped, pleomorphic	Circular dsDNA	Acholeplasma phage
Rudiviridae	Non-enveloped, rod-shaped	Linear dsDNA	Rudivirus
Fuselloviridae	Non-enveloped, lemon-shaped	Circular dsDNA	SSV-I
Inoviridae	Non-enveloped, filamentous	Circular ssDNA	Coliphage fd, MS2, ΦX174
Microviridae	Non-enveloped, isometric	Circular ssDNA	Spiroplasma phages
Leviviridae	Non-enveloped, isometric	Linear ssRNA	Coliphage QB
Cystoviridae	Non-enveloped, spherical	Segmented dsDNA	Φ6

The order *Caudovirales* comprises the vast majority (96%) of known phages. Members of the *Caudovirales* have DNA genome, head, and tail in common, as illustrated in Figure 1.4. The head has an icosahedral or elongated morphology. The tail is helical and generally provided with fixation structures (baseplates, spikes, and fibres) and serves as a DNA injection apparatus during the host infection process (Ackermann and Prangishvili, 2012). The morphological difference of the tail of *Caudovirales* member phages defines the three families of the order: *Myoviridae*, *Siphoviridae*, and *Podoviridae.* While the tails of *Myoviridae* and *Siphoviridae* are long and contractile, the tails of *Podoviridae* are short and non-contractile (Ackermann, 2009).

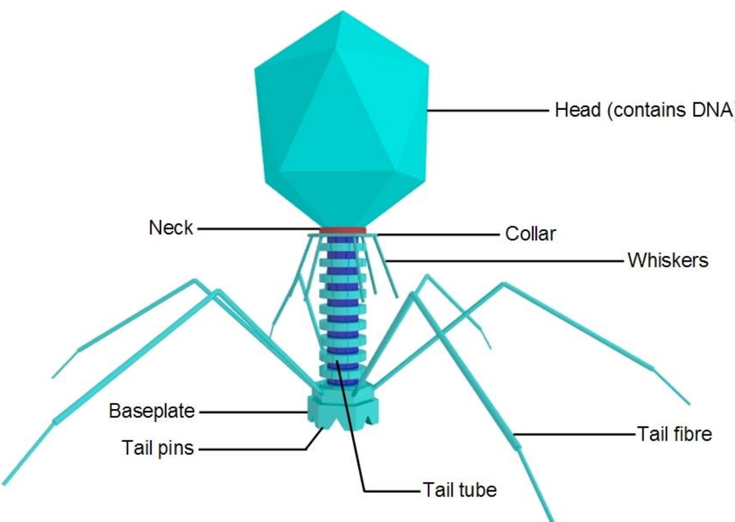

Figure 1. 4 Morphology of tailed bacteriophage (from (Nobrega *et al.*, 2018)).

Phages are the most abundant and diverse components of microbial communities (Kristensen *et al.*, 2013). It is estimated that about 10^{31} phages are present in the global biosphere, 10 times more genetic units than bacteria (Rohwer and Edwards, 2002). Phages can be found in places where bacteria or archaea are present, including humans, animals, foods, water, and soil (Kutter and Sulakvelidze, 2004). They are diverse in size, shape, structure, symmetry, and nucleic acid content (Ackermann, 2009).

Phages can be lytic (virulent) or lysogenic (temperate) depending on their life cycle, as shown in Figure 1.5. Lytic phages cause metabolism disruption and lysis of bacterial host cells soon after the initiation of infection, while lysogenic phages insert their genome into the bacterial genome. Lysogenic phages are mostly considered as horizontal gene transfer vectors, which may considerably contribute to the evolution of the bacterial host (Harper *et al.*, 2014; Gama *et al.*, 2013; Canchaya *et al.*, 2003).

1.7.1 Lytic phages

After locating the receptors, lytic phages penetrate the cell wall and inject their genetic material into the cytoplasm of the host cell. The genetic material of phages will be taken up by the host and divert cell biosynthetic machinery of the host for phage reproduction (Young,

1992a). Phage gene expression, genome replication, morphogenesis (formation of capsids and tails), and assembly and packaging of parts occur inside the host cell (Carlton, 1999). Once the production of phage virions in a good number or beyond the carrying capacity of host bacteria is completed, lysis of the host bacteria cell can be completed within few minutes using lytic enzymes such as a muralytic enzyme, or endolysin, and a holin (Paul *et al.*, 2011). The phage-encoded holins are transmembrane proteins that create lesions in the host cytoplasmic membrane through which endolysins gain access to the peptidoglycan layer (Young, 1992b). Additional membrane proteins called spanins are involved in the lysis process of the outer membrane in Gram-negative hosts by catalysing fusion of the inner and outer membranes (Young, 2014). Most phages use these enzymes to permeabilize the bacterial host membrane at a programmed time and thus control the length of the vegetative cycle. By contrast, single-stranded phages accomplish lysis by producing lysis protein without muralytic activity (Young *et al.*, 2000). Lysis of the host cell will result in the release of hundreds of fully matured virulent phage virions capable of infecting other host bacteria and initiate new phage lytic cycles (Richards, 2014).

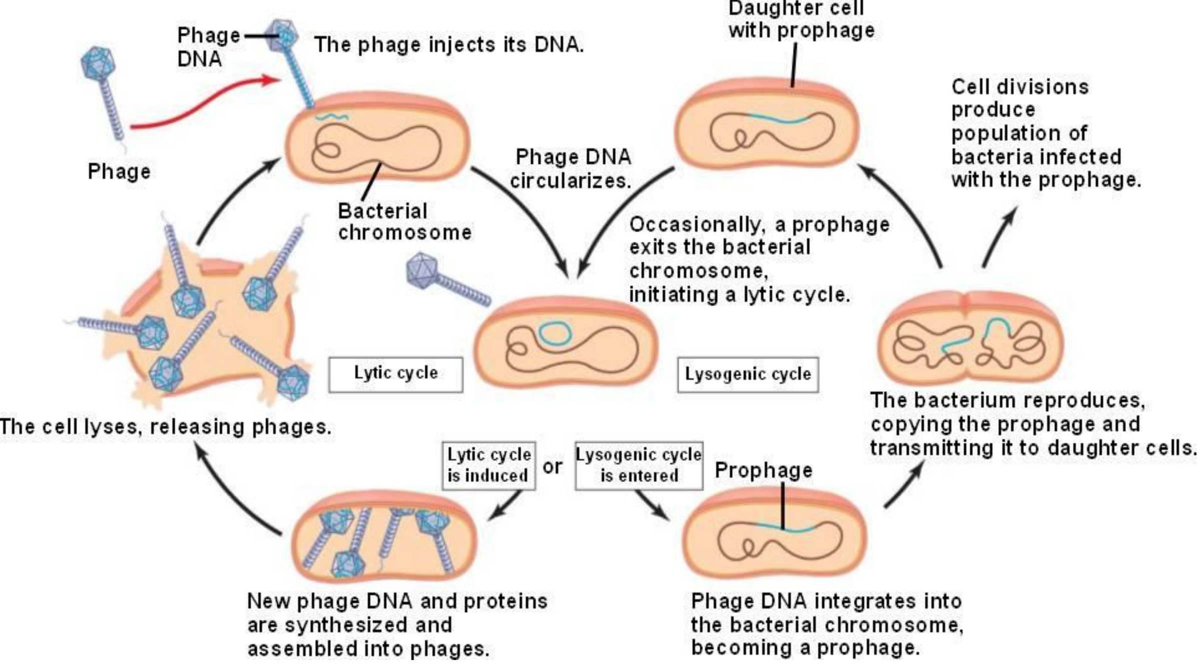

Figure 1. 5 Lytic and lysogenic life cycle of phages (from (Vander Elst and Meyer, 2018)).

1.7.2 Lysogenic phages

Upon successful entry to the bacterial host the same way as lytic phages, lysogenic phages integrate their genome into the host chromosome, with their genome in this state termed prophage. As a result, the phage genome stays dormant and replicates as part of the host, called lysogeny, and the genome is transmitted to a daughter cell when a host cell division occurs. Phages following this type of replication are also called temperate phages, and the host cells carrying a prophage are known as lysogenic cells (Oppenheim and Adhya, 2007). When a host bacterium is lysogenised, it may become resistant to infection by other related phages that share the same immunity group profile or repressor specificity (Davies *et al.*, 2016; Labrie *et al.*, 2010; Kenny *et al.*, 2006). However, due to stimuli such as environmental stress that bring bacterial host DNA damage, the prophage can be activated and induce lytic infection (Erez *et al.*, 2017; Oppenheim *et al.*, 2005). Lysogenic phages are not used for therapeutic purposes because they are inefficient in killing the bacterial host, or there is an increased risk of horizontal gene transfer that may lead to the transfer of virulence factors to the host (Davies *et al.*, 2016; Boyd and Brüssow, 2002).

1.8 The role of phages on human microbial communities

Phages have been recovered from diverse sites of the human body, including the skin (Hannigan *et al.*, 2015; Weyrich *et al.*, 2015), digestive tract (Edwards *et al.*, 2019; Lepage *et al.*, 2013; Mills *et al.*, 2013), respiratory tract (Willner *et al.*, 2009), and oral cavity (Pride *et al.*, 2012). Metagenomic studies on the microbiota of humans indicate that many of the viruses associated with healthy human tissues are phages (Hannigan *et al.*, 2015; Wylie *et al.*, 2014). A recent global study on human gut viruses showed that the phages termed crAssphages are widespread viruses strongly correlated with different clades of bacteria and an abundant benign component of the healthy human gut microbiome. The study confirmed

that crAssphages could be the reliable human fecal contamination markers signifying the diagnostic importance of phages (Edwards *et al.*, 2019). The study also lends strong support for the earlier discovery of crAssphage, which is more abundant than all other known phages together documented in the publicly available metagenomic data (Dutilh *et al.*, 2014).

Gut phages are believed to be individual specific as a result of the rapid evolution of phages and the complex gut microbiome dynamics (Minot *et al.*, 2011). Nevertheless, some phages in the gut might be highly conserved in people all over the world (Dutilh *et al.*, 2014). Phages are likely to produce a strong influence on the diversity and structure of bacterial communities of the human body either through predation or by supplying a pool of new, possibly beneficial genes (Hannigan *et al.*, 2015; Stern and Sorek, 2011; Breitbart *et al.*, 2003). They can also influence microbial diversity and structure by acting directly as immunomodulation agents (Hannigan *et al.*, 2015) and creating dysbiosis (De Paepe *et al.*, 2014). The switching of phages between lytic and lysogenic pathways also results in the evolution of bacterial hosts (Erez *et al.*, 2017; Klimenko *et al.*, 2016; Levin *et al.*, 2013).

How virulent phages avoid driving their bacterial prey to extinction despite being efficient predators is not yet elucidated (Heilmann *et al.*, 2012; Kim *et al.*, 2011). Lytic phages may be constraining factors for microbial community evolution because the lytic infection will decrease bacterial speciation rates by more than one order (Klimenko *et al.*, 2016; Levin *et al.*, 2013). Lysogenic infection may speed up bacterial evolutionary processes by extracting and inserting genetic materials into new hosts, shuffling genetic diversity in the bacterial hosts (Armstrong *et al.*, 2015). Some prophages may provide beneficial roles to their bacterial host, for example, by increasing biofilm formation capability and promoting colonization (Messad *et al.*, 2015). Hence, phage-bacteria interaction may act as a stabilizing factor for the microbiome through slowing down speciation by infecting the bacterial host

and creating a stable state of the microbiome community (Klimenko *et al.*, 2016; Maura and Debarbieux, 2012).

1.9 The use of phages as therapeutic agents

Since the discovery of phages as therapeutic agents in humans in 1919, the use of phage has been tested in plants, animals, and humans with various levels of success (Haq *et al.*, 2012). Phages have also been used for disease diagnosis, bacterial typing, and vaccine production (Sagona *et al.*, 2016; Tawil *et al.*, 2014). Studies have shown that phage therapy could be a potential alternative in combating antibiotic-resistant bacterial infections (Wittebole *et al.*, 2014; Górski and Weber-Dabrowska, 2005). Lytic phages can reach and lyse their host bacteria residing in biofilms (Olszak *et al.*, 2015; Saussereau *et al.*, 2014). Lytic phages could also be used as prophylactic agents against bacterial infections (Morello *et al.*, 2011). As phages are specific to their hosts, they show no or minimum off-target effects (Fu *et al.*, 2010). Phages are safe to apply in any route of administration (Pirnay *et al.*, 2015).

Some phages may have specificity for one or few host strains and others may infect different strains within a single species or multiple related bacteria species (Donlan, 2009; O'Flaherty *et al.*, 2005; Jensen *et al.*, 1998). The determining factor for the host range of phages is the specificity of receptors for the phage strain. A commonly described mechanism of bacterial resistance against phage attack is the prevention of phages from attaching to cell surface due to the absence or alteration of receptors (Hyman and Abedon, 2010). Preventing phage genome entry, degradation of phage genome by the restriction-modification (RM) and clustered regularly interspaced short palindromic repeats (CRISPR-cas) associated systems, preventing genome integration by super-infection exclusion systems (Sie), and blocking phage replication, transcription, translation, or virions assembly via the abortive-infection system are other bacterial resistance mechanisms to phage attack (Labrie *et al.*, 2010; Cairns

et al., 2009). The bacteria may also carry out apoptosis when infected with phage, which is part of an abortive infection (Labrie *et al.*, 2010; Chopin *et al.*, 2005).

Phage cocktails and engineered phages are preparations that could potentially improve the result of phage therapy (Bernasconi *et al.*, 2017; Alves *et al.*, 2016; Westwater *et al.*, 2003). High efficacy, bacterial host specificity, quick onset of action, and low risk of resistance, as in the case of phage cocktails, make phage therapy a feasible option for use in humans (Pastagia *et al.*, 2011). Phage cocktails have been found to exploit the synergistic effect of component phages, which could facilitate their adsorption and diffusion in multispecies biofilm infections (Chhibber *et al.*, 2015; Sillankorva *et al.*, 2010). When more than one types of phages from the phage cocktail attack the same bacterium, the phage cocktail may produce a better lytic effect than any single component phage due to synergy, or the mixture may be less effective than the best single phage because of phage interference (Schmerer *et al.*, 2014).

The advantages of phage cocktail over single phage are: a broad collective host range, preventing the development of phage-resistant bacterial mutant, and kill the bacterial population more rapidly or completely (Schmerer *et al.*, 2014). Hence, phage cocktail therapy is strongly recommended to effectively eliminate bacterial cells both in planktonic and biofilm states (Alves *et al.*, 2016).

1.10 Effect of phages on biofilms

Lytic phages have been found to produce enzymes that degrade bacterial capsule and biofilm extracellular polymeric substances (EPS) (Sutherland *et al.*, 2004). Phage-associated enzymes facilitate phage migration through mucoid biofilms (Sutherland *et al.*, 2004). With the help of these enzymes, phages move through biofilms and lyse bacterial host cell by endolysin

enzyme and holin and spanins proteins (Young, 2014). Then, phages eliminate their hosts via lytic activity (Zhang *et al.*, 2014b; Gilbert *et al.*, 2002). The weakening of the EPS and the capsule by phage-encoded enzymes facilitate phagocytosis or supports the effect of antibiotics (Morello *et al.*, 2011). These phage-associated enzymes are capable of degrading EPS of many genera (Pei and Lamas-Samanamud, 2014; Sutherland *et al.*, 2004). Phages with these depolymerase enzymes can readily reach the host cell surface by digesting their way and penetrate the bacterial cell membrane and cell wall by the help of endolysin, holin, and spanins or by contracting a sheath and injecting their genome into the cytoplasm of the host bacterium (Gonzalez *et al.*, 2017).

There is no consensus on whether phages maintain lytic efficacy in multispecies biofilms (Geredew Kifelew *et al.*, 2019). Some studies suggest that co-aggregation of bacterial cells and tight cell-to-cell binding in multispecies biofilms may occlude phage receptors (Kay *et al.*, 2011; Teplitski and Ritchie, 2009; Rickard *et al.*, 2003). As opposed to this, reports showed that most of the biofilms possess open architectures with water-filled channels that could allow phages to get access to the interior of the biofilms (Gutierrez *et al.*, 2015; Briandet *et al.*, 2008; Rickard *et al.*, 2003). It has been suggested that the presence of non-susceptible bacteria will not prevent phages from reaching and lysing their susceptible hosts in multispecies biofilms (Sillankorva *et al.*, 2010). Phage could be released either when the biofilms are sloughed-off because of aging, EPS degrading enzymes, hydrophobic and electrostatic interactions in membranes, or a combination of these factors (Sutherland *et al.*, 2004; Van Voorthuizen *et al.*, 2001). The high number of host bacteria within limited spaces in biofilms will facilitate phages movement from one host to the next (Harper *et al.*, 2014).

1.11 Features of phage therapy

Lytic phages could reduce the consequence of bacterial infection (Stratton, 2003). The process of phage infection and subsequent self-replication in bacteria offers advantages over antibiotics: phages amplify themselves at the infection site provided there are sensitive bacterial hosts (Wittebole *et al.*, 2014). The capability of phages to replicate in their bacterial host could reduce the number of repeated doses (Kelly *et al.*, 2012; Vieira *et al.*, 2012; Ryan *et al.*, 2011). Phages are safer as therapeutics than antibiotics because phages will only increase in density and remain in the body or environment as long as they get bacterial hosts (Mendes *et al.*, 2014; Kutter *et al.*, 2010; McVay *et al.*, 2007). Phages can evolve to overcome the resistance by previously susceptible bacteria (Levin *et al.*, 2013).

Limited studies indicated the importance of a phage cocktail in the treatment of multispecies biofilms that benefited from the synergistic effect of phages in facilitating phage adsorption and diffusion (Chhibber *et al.*, 2015; Sillankorva *et al.*, 2010; Schmerer *et al.*, 2014). The discovery of polyvalent phages affecting several species of bacteria shows the possibility of developing broader host range phages (Malki *et al.*, 2015; Kim *et al.*, 2012; Lin *et al.*, 2012; Sillankorva *et al.*, 2010; O'Flaherty *et al.*, 2005; Jensen *et al.*, 1998).

Phages do not cause the adverse side effects of antibiotics (Wittebole *et al.*, 2014). Some advantages of phages over antibiotics are (i) specificity to bacterial host that reduces damage to normal flora, (ii) self-limitation in the absence of bacterial host, (iii) equally effectiveness against antibiotic sensitive and resistant bacteria, (iv) better ability to disrupt bacterial biofilms, and (v) ease to isolate from the environment or select from a collection (Pirnay *et al.*, 2015; Golkar *et al.*, 2014; Wittebole *et al.*, 2014; Yosef *et al.*, 2014; Kutter *et al.*, 2010).

The human body is continually interacting with phages from the surrounding environment or human microbiome, and there is no evidence on phages' direct adverse effect (Edwards *et al.*, 2019; Loc-Carrillo and Abedon, 2011; Gorski *et al.*, 2003). There is evidence that phage therapy is safe to use in different routes of administration (Fong *et al.*, 2019; Ooi *et al.*, 2019; Drilling *et al.*, 2017). Therapeutic phages should be obligatory lytic and free from antibiotic resistance or bacterial virulence genes. Besides, phage cocktails should be affective against broad host range of the target pathogen without side-effect on non-target microbes and capable of complementation, in which resistant strains or mutants to one phage are lysed by other component phages (Law *et al.*, 2019; Casey *et al.*, 2018; Ceyssens *et al.*, 2009). The two-phage cocktails, AB-SA01 and AB-PA01, and their components used in this study satisfy these criteria and have entered clinical development. All components of each phage cocktail are well-characterized and produced under current good manufacturing practice (cGMP) standards (Lehman *et al.*, 2019; Fong *et al.*, 2019). Moreover, AB-SA01 and its components are approved by that complies with the U.S. Food and Drug Administration (FDA) and Australia's Therapeutic Goods Administration (TGA) to conduct clinical phase I trials and single-patient emergency treatment (Law *et al.*, 2019).

AmpliPhi Biosciences Corporation generously provided AB-SA01 and AB-PA01 phages as cocktails and their components. AB-SA01 is a combination of three *Myoviridae* phages designated J-Sa 36, Sa 83, and Sa 87 (Lehman *et al.*, 2019). AB-PA01 is a combination of Pa 193 and Pa 204 from *Myoviridae* and Pa 222 and Pa 223 from *Podoviridae* (Fong et al., 2017).

1.12 Significance of the study

Elements for the conception of this study are:

- A rapid rise of antibiotic-resistant infection is a big problem in DFU infection. The difficulty in developing new antibiotics for the last decade has prompted the search for new agents with effective antibiotic activity, including phages.
- There is evidence that phages can reduce bacterial biofilm biomass, but their efficacy against their host, mainly when it is found in multispecies biofilms has not been well addressed.
- DFU infection is polymicrobial and often involves antibiotic-resistant microbes, including *S. aureus* and *P. aeruginosa*. It is a chronic disease usually complicated with bacterial biofilms. Biofilms display heightened resistance to antibiotics. The limited information about the presence or absence of such heightened resistance by the target bacteria to phages in multispecies biofilms is the driving force for phage therapy development.
- Absence of approved broad-spectrum topical antibiotics and poor outcome of systemically administered antibiotics due to the widespread presence of MDR bacteria in polymicrobial DFU infections are compelling factors to study topically applied phage cocktails.

1.13 Objectives of the study

This research project was initiated to study the effect of phages in treating antibiotic-resistant infections of DFU with the following objectives.

- To evaluate the *in vitro* lytic efficacy and host range of phage cocktail AB-SA01 and its components against *S. aureus* clinical isolates in single-species planktonic and biofilm states.

- To examine the *in vitro* lytic efficacy and host range of phage cocktail AB-PA01 and its components on *P. aeruginosa* clinical isolates in single-species planktonic and biofilm cultures.
- To assess the efficacy of phage cocktails AB-SA01 and AB-PA01 against their host bacteria in mixed-species planktonic and biofilm cultures.
- To determine the effectiveness of topical AB-SA01 treatment in controlling antibiotic-resistant *S. aureus* infection and facilitating healing on a diabetic mice skin wound.

Chapter 2

Effect of Phage Cocktail AB-SA01 on Diabetic Foot Ulcer Clinical *Staphylococcus aureus* Isolates and Biofilms

Chapter 2: Effect of Phage Cocktail AB-SA01 on Diabetic Foot Ulcer Clinical *Staphylococcus aureus* Isolates and Biofilms

Abstract

The increasing incidence of infections caused by antibiotic-resistant bacteria such as methicillin-resistant and multidrug-resistant *Staphylococcus aureus,* a virulent pathogen infecting diabetic foot ulcer (DFU), requires alternative therapeutic agents such as phages with low cost, ready availability, minimum side effect, and high efficacy. This study examined the efficacy of three-phage cocktail AB-SA01, which is made to current good manufacturing practice (cGMP) standard, on *S. aureus* clinical isolates from DFUs. It was found that 94.3% of the isolates were susceptible to AB-SA01. AB-SA01 showed a stronger lytic efficacy than each of its components did, as measured with spot tests. AB-SA01 effectively decreased the biofilm biomass of *S. aureus* clinical isolates. Biofilms of isolates that were resistant during the spot test also showed reduced biofilm biomass reduction. A higher number of resistant biofilms were observed than the number of resistant isolates in spot tests, indicating biofilms and host cells in the biofilms are more resistant than in planktonic states for phage activity. The phage cocktail and its components were effective irrespective of the antibiotic susceptibility of the isolates. AB-SA01 is a potential treatment for antibiotic-resistant *S. aureus* DFU infections.

2.1 Introduction

2.1.1 *S. aureus* in DFU

Approximately one-in-three people living with diabetes will develop a foot ulcer, and most of these ulcers will become infected (Armstrong *et al.*, 2017; Neyra *et al.*, 2014; Zaine *et al.*, 2014). *S. aureus* remains a common and virulent cause of DFU infections (Richard *et al.*, 2011; Lipsky *et al.*, 2004). In some studies, close to 50% *S. aureus* isolates associated with DFU are MRSA (Tentolouris *et al.*, 2006; Zetola *et al.*, 2005). In most cases, *S. aureus* strains isolated from DFU have several virulence factors including toxins that play an important role in initiating inflammation and spreading the infection (Viquez-Molina *et al.*, 2018; Sotto *et al.*, 2008), exfoliatins which participate in skin invasion (Nhan *et al.*, 2011), and the *mec*A gene product that confers resistance to β-lactam antibiotics (Vu *et al.*, 2014). The effect of these virulence factors leads to deeper tissue infection, including bones and antibiotic treatment failure (Viquez-Molina *et al.*, 2018).

A quarter of DFU patients will undergo lower limb amputation due to antibiotic-resistant, often MDR, bacterial infections that result in treatment failure (Soge *et al.*, 2016; Brem *et al.*, 2004). Because clinical signs of systemic infections for example leukocytosis and fever are usually not manifested even in a severe cases (Armstrong *et al.*, 2017) and local symptoms and clinical signs of infection diminish due to concomitant peripheral neuropathy and ischemia (Ertugrul *et al.*, 2017), diagnosis and treatment of the DFU infection can be difficult. Therefore, amputation is considered a better outcome measure (Pickwell *et al.*, 2015). After the first lower limb amputation, up to 50% of patients require another amputation within three to five years (Armstrong *et al.*, 2007). Most patients with DFU suffer from a severe infection, an independent predictor of amputation, according to the

International Working Group on the Diabetic Foot (Bakker *et al.*, 2016; Pickwell *et al.*, 2015).

The prevalence of MDR infections in DFU is primarily associated with *S. aureus* (Aysert Yildiz *et al.*, 2018; Trivedi *et al.*, 2014). The failure of antibiotic therapy in some strains of *S. aureus,* such as small colony variants, could be because of the intracellular location and biofilm production capability (Cervantes-Garcia *et al.*, 2015). Identification of causative agents to the species level and the use of effective therapeutic agents after *in vitro* susceptibility tests are important for successful treatment of DFU (Abouhmad *et al.*, 2016; Lipsky and Hoey, 2009).

2.1.3 Identification of *S. aureus*

The commonly used biochemical identification methods of bacterial pathogens are time-consuming and often produce incomplete diagnosis (van Belkum *et al.*, 2013). The recently invented bacterial identification tool, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), is a robust, reliable, and simple compared to the traditional methods (Fondrie *et al.*, 2018; Perez-Sancho *et al.*, 2018; Harris *et al.*, 2010). MALDI-TOF MS uses soft laser ionization on bacteria or bacterial extracts to detect peptide and protein ions on the cell surfaces based on their relative masses and charges (Edwards-Jones *et al.*, 2000). Several studies have demonstrated the accuracy of MALDI-TOF MS for the identification of *S. aureus* (Perez-Sancho *et al.*, 2018; Elbehiry *et al.*, 2016; Spanu *et al.*, 2011; Harris *et al.*, 2010); hence used in this study.

2.1.4 Antibiotic susceptibility testing of *S. aureus* isolates

A short turn-around time of antibiotic susceptibility testing is important in improving clinical outcomes of bacterial infectious disease patients through the provision of appropriate and

timely antibiotic treatment (Ha *et al.*, 2018). Modern laboratories worldwide use an automated system for rapid antibiotic susceptibility testing such as the VITEK® 2 to test bacteria, including *S. aureus* (Ligozzi *et al.*, 2002; Horstkotte *et al.*, 2002). VITEK® 2 uses analysis of minimum inhibitory concentration (MIC) patterns using Advanced Expert System (AES) software. The VITEK® 2 system demonstrated acceptable accuracy for a range of antibiotics against *S. aureus* isolates (Nonhoff *et al.*, 2005). According to the National Committee for Clinical Laboratory Standards (NCCLS) breakpoints, VITEK® 2 can provide up to 94 - 100% correct category agreement for *S. aureus* (Bobenchik *et al.*, 2014; Ligozzi *et al.*, 2002).

2.1.5 Phages as an alternative treatment for *S. aureus* infection

The prevalence of MDR *S. aureus* infections (Gupta and Prasad, 2011) and the absence of new antibiotics necessitate the discovery and development of new antimicrobial agents such as phages (Sotto *et al.*, 2008). Lytic phages are potential alternatives to antibiotics because they can kill host bacteria irrespective of their antibiotic susceptibility (Gu *et al.*, 2011). As reviews on the efficacy of lytic phages to control bacterial infections as a replacement or supplement of antibiotics showed (Golkar *et al.*, 2014; Maura and Debarbieux, 2011; Kutateladze and Adamia, 2010; O'Flaherty *et al.*, 2009), development of resistance by bacterial host against phages can be prevented or delayed by using lytic phage cocktails that target diverse bacterial cell surface receptors (Gutierrez *et al.*, 2018; Estrella *et al.*, 2016). Staphylococcal lytic phages have the potential as a therapeutic agent of human infections (Ceyssens *et al.*, 2009).

2.1.6 Phage cocktail to treat *S. aureus* infection

Most of the phages used for therapy are obligatory lytic, possess a proteinaceous tail, as illustrated in Figure 2.1, and are from *Myoviridae*, *Siphoviridae*, or *Podoviridae* (Gill and Hyman, 2010). A phage or a mixture of phages must have the capability of infecting a wide variety of bacterial host strains (Alves *et al.*, 2014; Synnott *et al.*, 2009; O'Flaherty *et al.*, 2005). Development of phage cocktails is one way to broaden the host range of phages (Manohar *et al.*, 2019) and delay phage resistance development (Alves *et al.*, 2016; Born *et al.*, 2011). Phage cocktail AB-SA01, used in the current study, is a fixed-composition of three naturally occurring and obligately lytic myoviruses related to Staphylococcus phage K. AB-SA01 is intended to treat *S. aureus* infections. It is produced under current good manufacturing practices, and characteristics of the component phages meet regulatory and generally accepted criteria for human use (Law *et al.*, 2019).

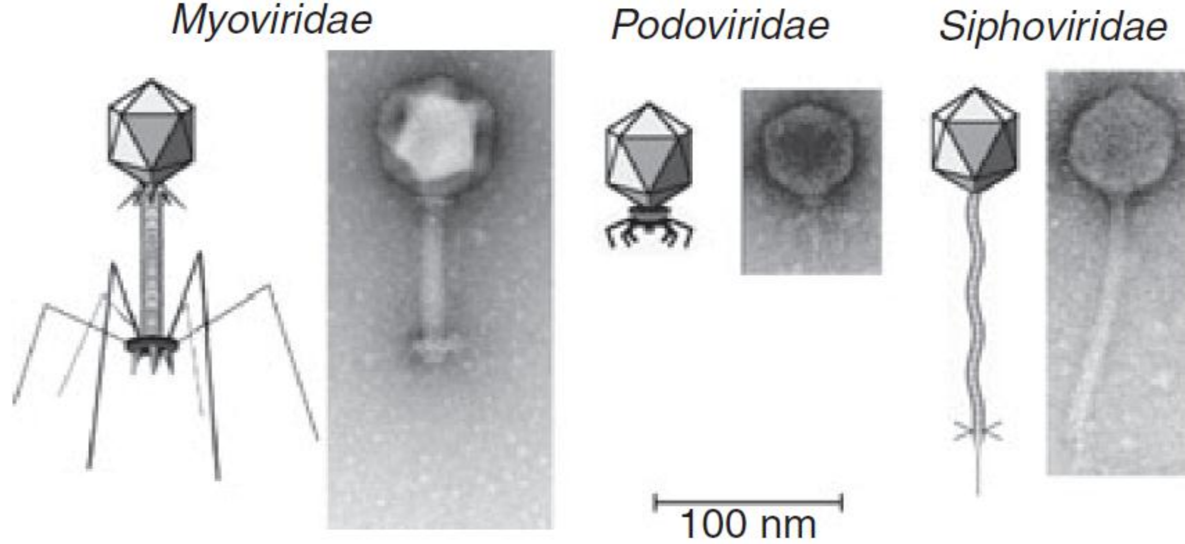

Figure 2. 1 Tailed phages (from (Harper and Enright, 2011)).

2.2 Materials and methods

2.2.1 *S. aureus* isolates

Bacterial isolates presumed to be *S. aureus* were collected from DFU patients seen in diabetic clinics in various hospitals in the Adelaide metropolitan area, South Australia. All specimens were identified using a previously described protocol (Mendes *et al.*, 2013; Blair *et al.*, 1967; Chapman, 1945). South Australia Pathology generously provided the isolates on Muller Hinton agar plates. A colony of each isolate was taken and platted on selective agar for *S. aureus*, mannitol salt agar (MSA) (Thermo Fisher Scientific, Victoria, Australia). After 24 hours of incubation aerobically at 37 °C, yellow or golden colonies surrounded by yellow zones were considered *S. aureus*. After 24 hours incubation at 37 °C, cultures on MSA plates were stored at 4 °C until further analysis.

2.2.2 Identification of *S. aureus* isolates

At about 18 hours of growth of the presumed *S. aureus* isolates on MSA, an isolated colony or a portion of it was smeared onto the sample spot of a stainless steel MALDI-TOF MS target. A thin film was formed by spreading the bacterial cells evenly using a sterile inoculation stick. One microliter of 70% formic acid was applied to the samples and left to dry at room temperature. Samples were then overlaid with 1 µl of the alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix solution and allowed to dry in a fume hood before putting the sample into the Bruker Daltonik MALDI-TOF MS Biotyper (BRUKER Pty. Ltd., Victoria, Australia) to identify bacterial species as described (Lee *et al.*, 2015; Schumann and Maier, 2014; Lasch *et al.*, 2014; Harris *et al.*, 2010). The results were analyzed using Biotyper 3.0 software. Isolates were considered *S. aureus* when both the first- and second-

best match organism scores were ≥ 2.00. Reference strains *S. aureus* subsp. *aureus* Rosenbach (ATCC 6538) and *S. aureus* subsp. *aureus* RN4220 were used for quality control. Isolates were stored in 25% glycerol nutrient broth at -150 ^{O}C until further analysis.

To quickly confirm any doubtful isolates, the latex agglutination test using a Staphaurex* (Thermo Fisher Scientific, South Australia, Australia) was done following the manufacturer's instructions. In summary: a drop of Staphaurex* reagent was dispensed onto a reaction card, a colony was picked and mixed with the Staphaurex*, and any resulting agglutination was examined after rotating the card gently for about 20 seconds. The development of agglutination was considered a positive result, whereas no agglutination was taken as a negative result.

2.2.4 Antibiotic susceptibility of *S. aureus* isolates

Following the manufacturer's instructions, *S. aureus* isolates were diluted in 0.45% sterile saline solution and standardized between 0.5 and 0.63 McFarland turbidity standards. Next, 280 µl of the bacterial suspension was transferred into 3 ml of a 0.45% saline solution tube, and the tube was placed in the cassette with a susceptibility card in it. The antimicrobial susceptibility test card loaded tube was incubated in the VITEK® 2 machine (bioMérieux Australia Pty Ltd, New South Wales, Australia) for overnight analysis as described earlier (Weber *et al.*, 2017; Gardiner *et al.*, 2013; Cartwright *et al.*, 2013; Ligozzi *et al.*, 2002). A loopful of the suspension was also cultured on blood agar overnight at 37 °C to check contamination during sample handling. Antibiotics included in the susceptibility test card (VITEK® 2 AST-P656) were benzylpenicillin, oxacillin, gentamicin, ciprofloxacin, erythromycin, clindamycin, linezolid, daptomycin, teicoplanin, vancomycin, tetracycline, nitrofurantoin, fusidic acid, mupirocin, rifampicin, and trimethoprim-sulfamethoxazole

(TSM). The VITEK® 2 system software 8.01 was used to produce antibiotic susceptibility profile data.

2.2.5 Determination of phage titer

Phage cocktail AB-SA01 and each of its phage constituents (J-Sa 36, Sa 83, and Sa 87) were provided by AmpliPhi Biosciences Corporation. Phage solutions were transported and stored under +4 ^{o}C temperature. All phages were from the *Myoviridae* family, and they were characterized by whole-genome sequence analysis and proved free from bacterial virulence or drug-resistance genes. Moreover, AB-SA01 complied with the U.S. Food and Drug Administration (FDA) and Australia's Therapeutic Goods Administration (TGA) requirements to conduct clinical trials and single-patient emergency treatment (Lehman *et al.*, 2019).

Phage titer was determined using double agar overlay plaque assays as described previously (Mirzaei and Nilsson, 2015; Merabishvili *et al.*, 2009) on susceptible two laboratory strains and two clinical isolates of *S. aureus*. Each phage and the phage cocktail titers were examined in triplicate. For estimating the initial phage concentration, the average number of plaques forming units (PFU) was taken, and the phage titer in terms of PFU in 1 ml phage suspension was calculated using the equation: $pfu/ml = \frac{Number of\ plaques}{d \cdot V}$ where d stands for dilution factor and V for the volume of inoculum. The mean was then calculated for the triplicate plates.

2.2.6 Phage efficacy and host range test

To evaluate the lytic efficacy and host range of phages, spot test was performed based on the previously described procedure (Mirzaei and Nilsson, 2015; Alves *et al.*, 2014). Briefly, 100

μl of an overnight culture of each bacterial isolate was mixed with 3 ml of tryptic soy soft agar that was warmed at 42 ^{o}C and poured onto pre-warmed at 37 ^{o}C tryptic soy agar (TSA) plates. Plates were rotated to ensure even distribution of the soft agar and left to dry at room temperature for 30 minutes. Ten microliters of each phage solution standardized to a titer of 9 log_{10} (PFU/ml) was serially diluted with tryptic soy broth (TSB). Ten microliters of 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7} dilutions were spotted onto the bacterial lawns in triplicate. Sterile PBS was used as a negative control. Plates were incubated inverted at 37 ^{o}C aerobically overnight and examined the following day. Based on the AmpliPhi Biosciences Corporation instruction, the criteria used to assess susceptibility were: (i) resistance if a very week or no activity is seen, (ii) intermediate when faint and turbid spots or partial activity is observed, and (iii) susceptible when clear spot or full activity with no bacterial growth within the spot is observed.

2.2.7 Measurement of the effect of phage cocktail on *S. aureus* biofilm

Biofilms of two laboratory strains and 52 clinical isolates of *S. aureus* were established in flat-bottom 96-well microtiter plates after 48 hours incubation at 37 ^{o}C following the established model and measured using optical density at 600_{nm} (Avila-Novoa *et al.*, 2018; Kouidhi *et al.*, 2010; Rohde *et al.*, 2007). Isolates were assigned based on recommended criteria as: (i) optical density $(OD)_{600} < 0.1$ non-, (ii) $0.1 \leq OD_{600} < 1$ weak-, and (iii) $OD_{600} \geq 1$ strong-biofilm producers. Phage cocktail treatment was applied as described (Gonzalez *et al.*, 2017; Thomas *et al.*, 2015; Mendes *et al.*, 2014) with the following modifications. *S. aureus* isolates were cultured overnight on MSA at 37 ^{o}C. A single colony from each isolate was transferred into a sterile glass tube of 0.45% sterile saline and adjusted to 1.0 McFarland turbidity standard. The suspension was diluted at 1:100 into 1% glucose-TSB, and 150 μl of the suspension was transferred to a sterile flat-bottom 96-well Greiner CELLSTAR®

polystyrene tissue culture plate (Sigma-Aldrich, New South Wales, Australia). Plates were incubated for 48 hours on a gyratory mixer of 70 revolutions per minute (rpm) at 37 ^{o}C.

Following incubation, the liquid part of the culture was removed, and the plates were washed three times with sterile deionized water through gentle pipetting. After the biofilms are air-dried, 180 µl of phage, tetracycline (16 µg/ml, positive control), or sterile PBS (negative control) solutions in broth were applied. The treatment broths were: (i) 18 µl phage solution (at 9 log_{10} (PFU/ml)) mixed with 162 µl broth, (ii) 3 µg tetracycline in 180 µl broth, and (iii) 18 µl PBS in 162 µl broth. After 12 hours of incubation in a static condition, the fluid portion of the culture was decanted, and the biofilm was washed twice through gently immersing into distilled water and inverting onto a paper towel. The biofilms were then fixed using 95% methanol for 30 minutes. The methanol was decanted, and the plates were washed once with sterile distilled water and air-dried.

The dried biofilms were stained with 190 µl of 0.2% crystal violet (CV) per well for 60 minutes. The excess stain was removed by gentle washing twice with distilled water, and the stained biofilm was left in a dark room overnight to dry. The stained biofilm was eluted in 200 µl of 30% acetic acid for 30 minutes at room temperature. The eluted suspension was transferred into a new micro-plate using 30% acetic acid as a negative control, and OD measurement was performed at 600_{nm} using a FLUOstar® Omega multi-mode microplate reader (BMG LABTECH Pty. Ltd. Victoria, Australia). The OD reading data was analyzed and compared with the mean of the negative controls. The percentage of biofilm biomass reduction designated as %BK was calculated from the absorbance of background-corrected untreated controls (I_C, PBS treated) and the absorbance of the treatments (I_T) as indicated previously (Thomas *et al.*, 2015):

$$\%BK = \frac{I_C - I_T}{I_C} \times 100\%$$

2.2.8 Measurement of the effect of phage cocktail on *S. aureus* isolates in biofilms

Measurement of the effect of phage cocktail treatment on *S. aureus* isolates in biofilms was conducted through colony count following the established procedure (O'Toole, 2016; Gutierrez *et al.*, 2015; Mendes *et al.*, 2013; Sillankorva *et al.*, 2010). On the 48 hours biofilm prepared as above, phage cocktail and control treatments were applied for 12 hours. Next, the treated biofilms were washed twice through gentle immersing into distilled water, and the washing water was removed by inverting onto a paper towel for a few minutes. The biofilm-associated cells were collected with 190 µl TSB using pipette after scrapping the bottom and wall of the wells with a loop. For the complete disruption of the biofilm and detachment of the cells, the TSB suspension was pipetted up and down 10 times and homogenized with a vortex mixer.

The phage-treated wells cell suspensions were serially diluted in a virucide solution, ferrous ammonium sulfate (FAS) (McNerney *et al.*, 1998), to inactivate free phages. FAS solution was prepared at 10 mM concentration immediately before use and filter sterilized using 0.22 µm pore size. After 15 minutes incubation at room temperature, 100 µl of serially diluted solutions were mixed with 3 ml of tryptic soy soft agar warmed at 42 ^{o}C and poured onto pre-warmed at 37 ^{o}C TSA plates. Treatment control wells also passed through a similar process except for FAS treatment. After 24 hours of incubation at 37 ^{o}C, colony count was performed, and the number of bacterial cells calculated. Three independent experiments were performed in triplicate. This was conducted on randomly selected one laboratory strain and six clinical isolates of *S. aureus*. Plates with an estimated 30 - 300 colonies were selected for colony count. The mean colony-forming unit (CFU) of the three plates of the same dilution was taken as final. The number of bacterial cells was calculated using the formula B = N/d

where B = number of bacteria; N = average number of colonies; and d = dilution factor as previously established (Mendes *et al.*, 2013).

2.2.9 Data management and statistical analysis

Data were double entered, encoded, and stored using Microsoft Excel Spreadsheet. STATA version 16 software was used for statistical analysis. All results are reported as the mean ± standard error of the mean and are compiled from at least three separate experiments and are expressed as logarithm-transformed values $\log_{10}$ (CFU /ml) over time. A comparison of experimental groups was performed using a one-way analysis of variance (two-tailed) or paired 't-test'. A $p < 0.05$ value was considered statistically significant.

2.3 Results

2.3.1 Isolation of *S. aureus* isolates using culture

The presumed *S. aureus* isolates were subcultured on MSA. Large yellow colonies surrounded by yellow zones on MSA, as shown in Figure 2.2, which confirmed mannitol fermentation after 24 hours of aerobic incubation at 37 °C, were considered *S. aureus* colonies. A total of 54 clinical *S. aureus* isolates were isolated and stored at -150 ºC in 25% glycerol until further use.

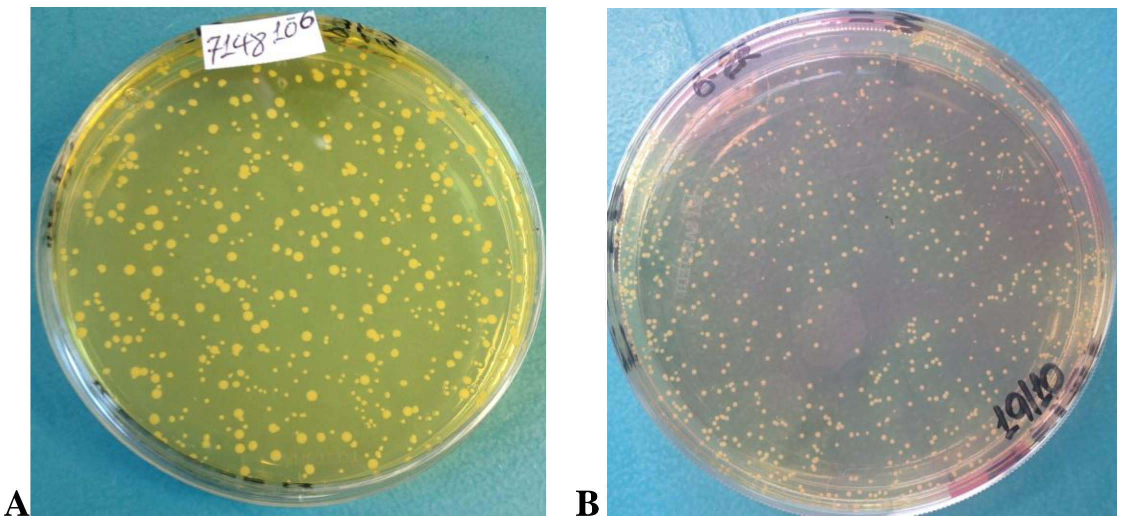

Figure 2. 2 Yellow *S. aureus* colonies and zone around the colonies on MSA (A and B)

2.3.2 Identification of *S. aureus* isolates using MALDI-TOF MS

All the 54 *S. aureus* isolates sorted by colony characteristics on MSA were examined using MALDI-TOF MS as above, and the 52 isolates were identified as *S. aureus.* The remaining two isolates were *Staphylococcus epidermidis* and *Staphylococcus lugdunensis*. Quality control was performed using *S. aureus* subsp. *aureus* Rosenbach (ATCC 6538) and *S. aureus* subsp. *aureus* RN4220 reference strains.

2.3.3 Identification of *S. aureus* using latex agglutination test

Isolates that showed different colony characteristics on MSA during subsequent cultures after identified as *S. aureus* through MALDI-TOF MS were also confirmed using the latex agglutination test, as shown in Figure 2.3. The five clinical isolates with doubtful colony characteristics on MSA were confirmed *S. aureus.*

Figure 2. 3 Positive latex agglutination test to identify *S. aureus.*

2.3.4 Antibiotic susceptibility of *S. aureus* isolates using VITEK® 2 test

VITEK® 2 was used to read MICs through the analysis of bacterial growth with antibiotics in test cards. The result indicated that all the 54 tested *S. aureus* isolates, including laboratory strains, are susceptible to gentamicin, nitrofurantoin, fusidic acid, mupirocin, and rifampicin. Besides, 98.2% (n = 53) of the isolates were susceptible to linezolid, tetracycline, and trimethoprim-sulfamethoxazole (TSM), as shown in Figure 2.4. The highest antibiotic resistance was seen against benzylpenicillin followed by erythromycin, oxacillin, and clindamycin with 77.8% (n = 42), 20.4% (n = 11), 18.5% (n = 10), and 18.5% (n = 10) resistant isolates, respectively.

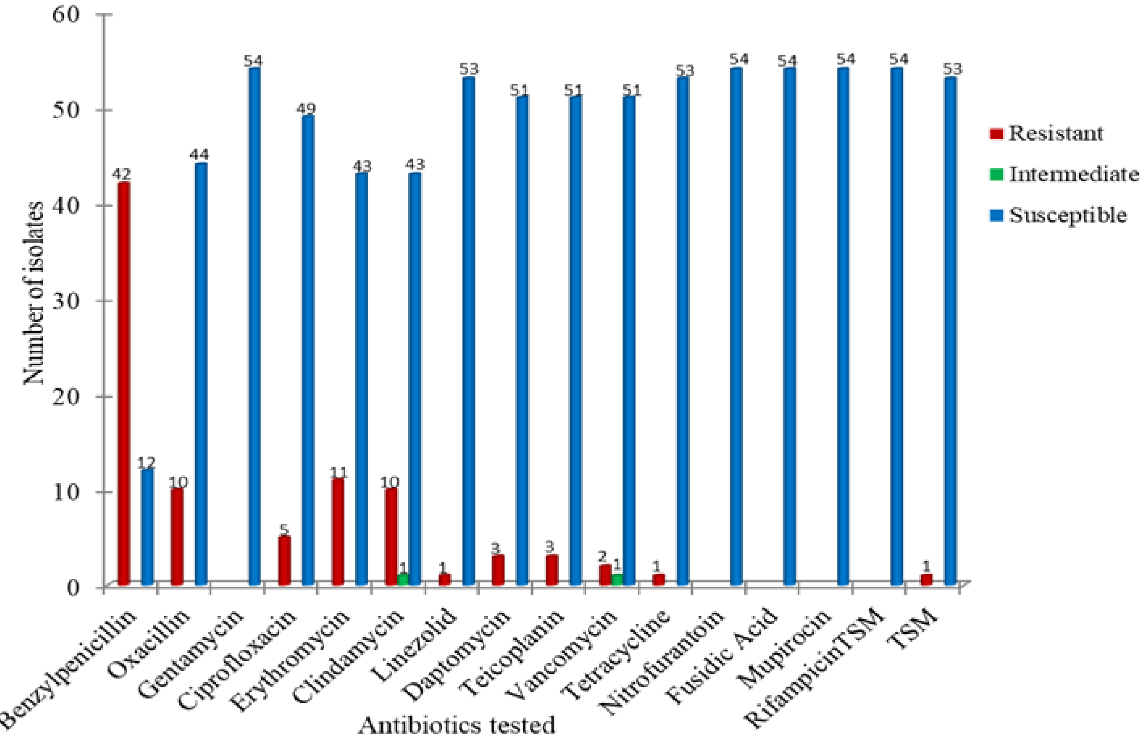

Figure 2. 4 Antibiotic susceptibility profile of *S. aureus* isolate used in this study

The antibiotic susceptibility test also showed that 6.7% (n = 9) of the isolates were MRSA, as shown in Figure 2.5. Worryingly, 13% (n = 7) of the isolates were MDR, resistant to three or more antimicrobial classes.

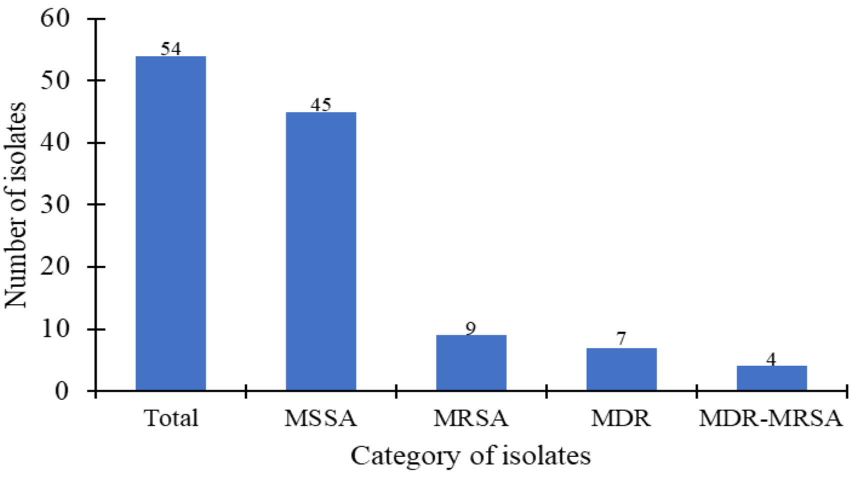

Figure 2. 5 Category of *S. aureus* due to their antibiotic susceptibility profile: MSSA – methicillin-susceptible *S. aureus*, MRSA – methicillin-resistant *S. aureus*, MDR – multidrug-resistant *S. aureus*, and MDR-MRSA – multidrug- and methicillin-resistant *S. aureus*.

2.3.5 Determination of phage titer

The phage titer test was conducted on susceptible two laboratory strains (RN4220 and SA6538) and two clinical isolates of *S. aureus* (63-2498 and 63-6378), as illustrated in Figure 2.6. The mean titer, presented in plaque-forming unit/millilitre - PFU/ml, was 9.3 $\log_{10}$ (PFU/ml) for J-Sa-36 and Sa-83, 9.0 $\log_{10}$ (PFU/ml) for Sa-87, and 9.1 $\log_{10}$ (PFU/ml) for the combined product AB-SA01. The result showed a mean of 9.2 $\log_{10}$ (PFU/ml) for all the three *S. aureus* phages (J-Sa 36, Sa 83, and Sa 87) used in this study**.** This phage titer was similar to the AmpliPhi Bioscience Corporation evaluation, which was 9.3 $\log_{10}$ (PFU/ml) for J-Sa 36 and Sa 83, and 9.0 $\log_{10}$ (PFU/ml) for Sa 87.

Figure 2. 6 Plaques of AB-SA01 on *S. aureus* RN4220

2.3.6 Efficacy of phages on *S. aureus* isolates

In evaluating the lytic efficacy and the host range of phage cocktail AB-SA01 and its components, a total of 53 *S. aureus*, 51 clinical isolates, and two laboratory strains were spot tested. The result showed that J-Sa 36, Sa 83, Sa 87, and AB-SA01 effectively lysed 75.5% (n = 40), 80% (n = 44), 94.3% (n = 50), and 88.7% (n = 47), respectively, of the isolates with full or partial activity as illustrated in Figure 2.7. Although Sa 87 lysed a greater number of isolates than AB-SA01, the difference was not statistically significant ($p > 0.05$; 94.3% vs 88.7%).

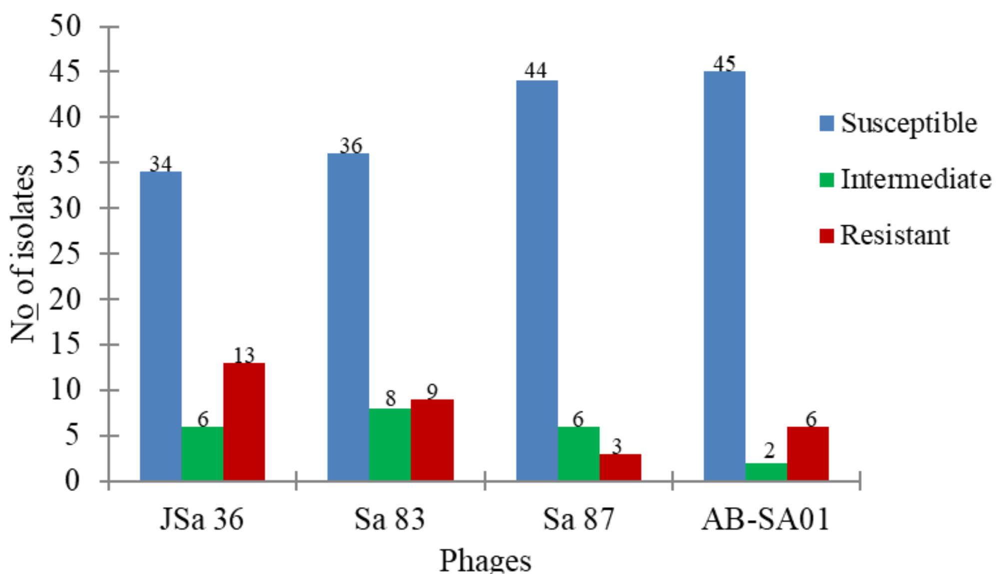

Figure 2. 7 Lytic efficacy and host range of AB-SA01 and its components on *S. aureus* isolates.

Clear full lysis was observed in 95.8% (n = 45/47), 88% (n = 44/50), 85% (n = 34/40), and 81.8% (n = 36/44) of the susceptible isolates to AB-SA01, Sa 87, J-Sa 36, and Sa 83, respectively, as shown in Figure 2.8. Two *S. aureus* clinical isolates were resistant for all the phages, including the phage cocktail AB-SA01. All the isolates that were resistant against AB-SA01 were either resistant or intermediately susceptible (partial lysis) to all component

phages. All MDR, MRSA, and MDR MRSA isolates, except two MDR MRSA, were susceptible to AB-SA01. There was no statistically significant difference in phage susceptibility ($p > 0.05$) among antibiotic-susceptible (89.5% AB-SA01 susceptible) and antibiotic-resistant (85.7% AB-SA01 susceptible) isolates.

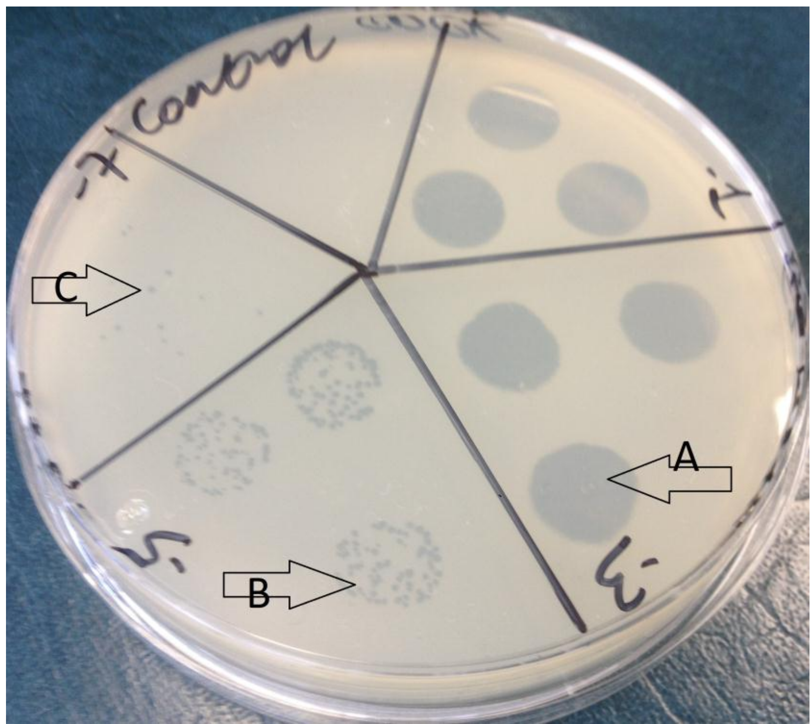

Figure 2. 8 Lytic activity of AB-SA01 on *S. aureus* isolates: **A** complete, **B** intermediate, and **C** no lysis.

2.3.7 Evaluation of the effect of AB-SA01 on *S. aureus* isolates biofilm

The effect of AB-SA01 on established biofilm biomass was studied and quantified using the CV staining method, as shown in Figure 2.9. Based on the criteria set in the methods section, 92.5% (n = 49) and 7.5% (n = 4) isolates were weak- and strong-biofilm producers, respectively. One isolate failed to produce a measurable biofilm.

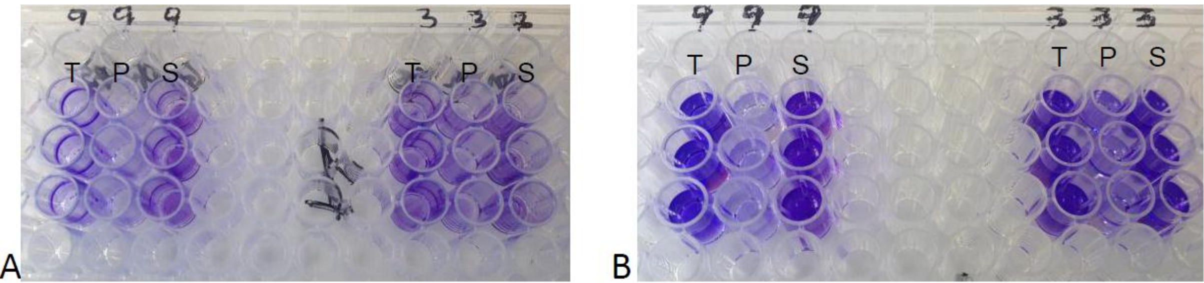

Figure 2. 9 Quantification of biofilm**:** **A** - CV stained biofilms (T, P, and S represent tetracycline, phage, and PBS treated group, respectively), and **B** - eluted solution of the stained biofilms for OD_{600nm} reading.

The established biofilms were treated with AB-SA01 suspension at one multiplication of infection (MOI) based on the result of a pilot test to determine bacterial density in 48 hours biofilm. After 12 hours of treatment, the biofilm biomass reduction due to AB-SA01 treatment was compared with PBS and tetracycline (TTC) treatment on seven randomly selected isolates biofilms. The biofilm biomass reduction was categorized: (i) insignificant; $<$ 20%, (ii) weak; 20.1- 40.0%, (iii) moderate; 40.1- 60.0%, (iv) strong; 60.1- 80.0 %, and (v) very strong $>$ 80 %. Based on these criteria, 20.8% (n = 11), 15.0% (n = 8), 17.0% (n = 9), 41.5% (n = 22), and 5.7% (n = 3) of the isolates' biofilms showed insignificant, weak, moderate, strong, and very strong biomass reduction, respectively, as shown in Figure 2.10. Biofilms developed from five susceptible *S. aureus* isolates did not show significant biomass reduction because of AB-SA01 treatment.

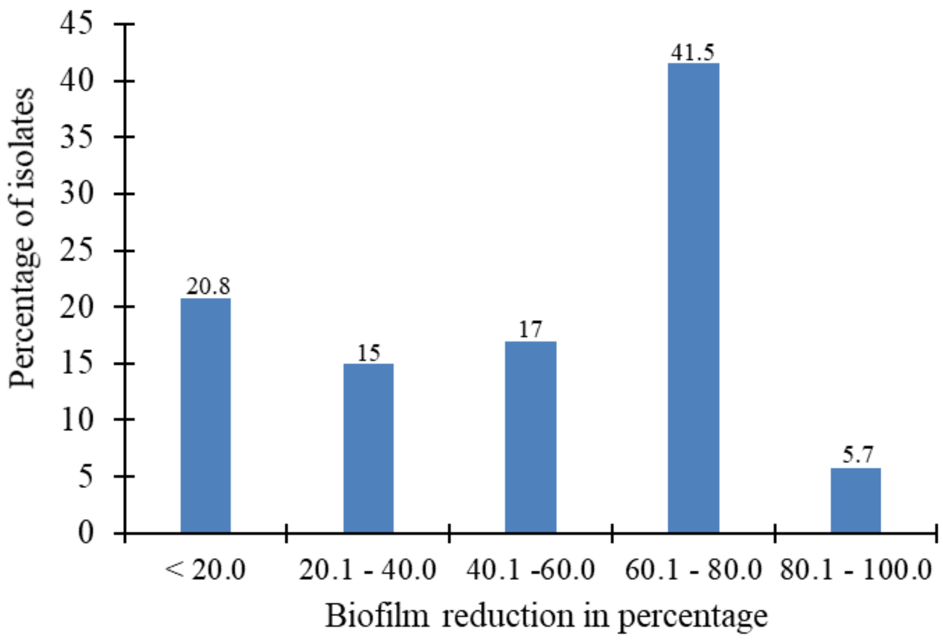

Figure 2. 10 Effect of AB-SA01 on *S. aureus* isolates biofilm.

Eighty percent of the biofilms showed a statistically significant reduction in biomass ($p < 0.05$; 0.13 vs 0.34) as a result of AB-SA01 treatment regardless of the antibiotic susceptibility profile of the isolates. There was no statistical significance difference in biofilm biomass reduction ($p > 0.05$; 0.18 vs 0.16) between antibiotic-resistant and antibiotic-susceptible *S. aureus* isolates. The maximum biomass reduction due to AB-SA01 treatment was 91.7% in laboratory strains and 81.3% in clinical isolates. The tetracycline treated biofilms also showed statistically significant biomass reduction ($p < 0.05$; 0.09 vs 0.26). In this study, there was no statistically significant difference ($p > 0.05$; 0.07 vs 0.09) between AB-SA01 and tetracycline treatment in reducing the biofilm biomass of *S. aureus* isolates, as illustrated in Figure 2.11.

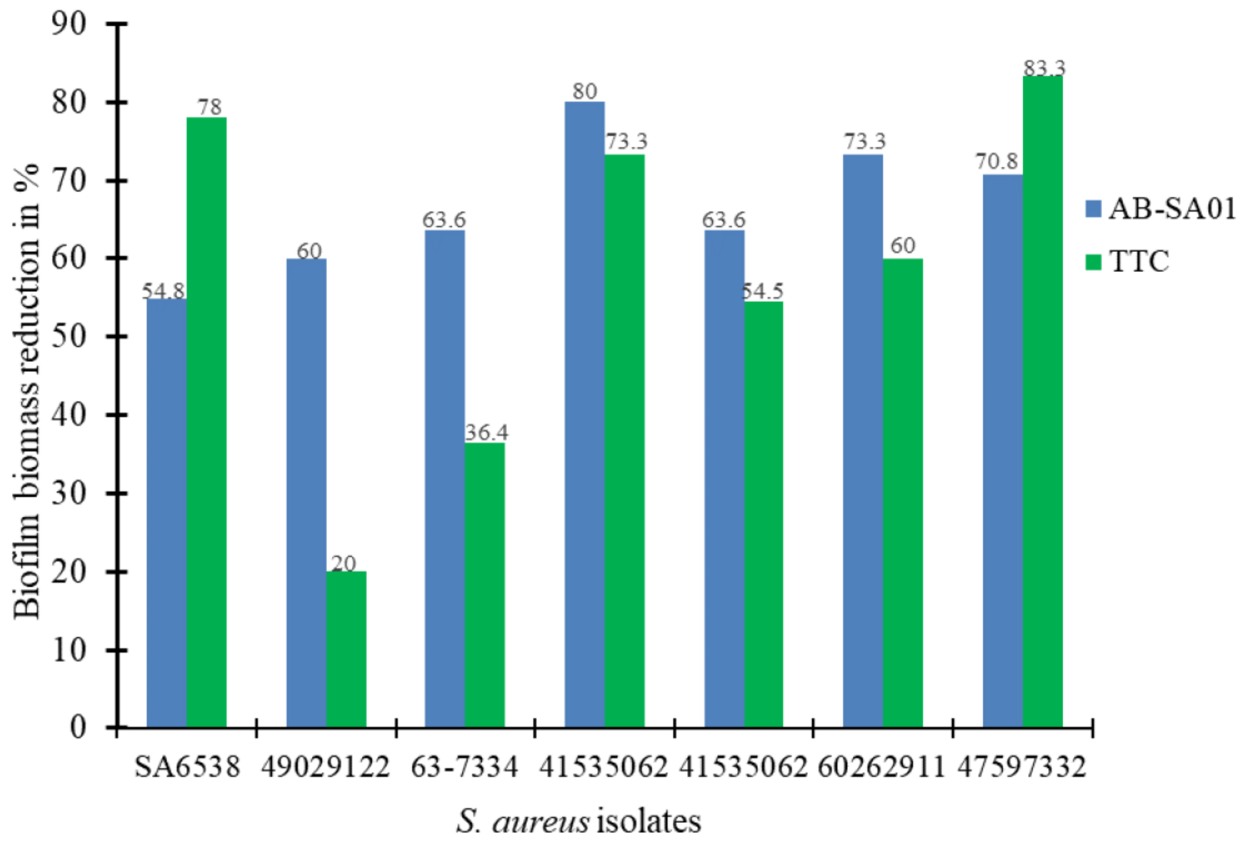

Figure 2. 11 Comparison of biofilm biomass reduction between AB-SA01 and tetracycline (TTC) treatments.

2.4 Discussion

S. aureus abundantly grows on MSA with large colonies surrounded by yellow zones, but other staphylococci produce small pink to red colonies surrounded by red or purple zones while other bacteria are inhibited (Blair *et al.*, 1967; Chapman, 1945). It was noted that *S. aureus* isolates tested in this study showed the colony characteristics of *S. aureus* discussed above on MSA. A few isolates that showed small yellow colonies surrounded by weak yellow zones were further confirmed by latex test based on the criteria for the identification of *S. aureus.* The finding of *S. aureus* identification using latex test in the current study agreed with previous reports (Smole *et al.*, 1998; Fournier *et al.*, 1993; Qadri *et al.*, 1991).

Diagnostic laboratories have widely used MALDI-TOF MS for bacterial identification. MALDI-TOF MS is a rapid, high throughput, reliable, and direct colony method (Tracz *et al.*, 2018; Dingle and Butler-Wu, 2013; Harris *et al.*, 2010; Lay, 2001). Using MALDI-TOF MS, 52 *S. aureus* clinical isolates were identified in the current study from 54 presumed *S. aureus* isolates. Findings similar to this study results are available in studies conducted to confirm the suitability of MALDI-TOF MS in identifying *S. aureus* at the species level (Perez-Sancho *et al.*, 2018; Camoez *et al.*, 2016; Lasch *et al.*, 2014; Harris *et al.*, 2010). A limitation to the use of MALDI-TOF MS is that it cannot identify bacteria to strain level. Hence, advanced techniques such as sequencing are recommended to strain level identification.

Although it is difficult to compare antibiotic-resistance data due to differences in sample size and study designs, a recent report of a high number of antibiotic-resistant *S. aureus* isolates circulating in the community is worrying (Haddad Kashani *et al.*, 2018). In this study, a significant proportion, 16.7%, of *S. aureus* isolates were MRSA, which agreed with previous reports (Ashong *et al.*, 2017; Bessa *et al.*, 2015). However, this finding is lower compared with 50% - 60% MRSA reports (Reveles *et al.*, 2016; Trivedi *et al.*, 2014; Tentolouris *et al.*,

2006). Recent reports show that MRSA isolates are quickly becoming MDR *S. aureus* (Soge *et al.*, 2016; Kim and Jeon, 2016). In this study, more than half of the MRSA isolates were MDR *S. aureus.* This observation was consistent with the previous study findings on wound infections (Bessa *et al.*, 2015; Trivedi *et al.*, 2014; Galkowska *et al.*, 2009).

In line with earlier reports (Uçkay *et al.*, 2015; Diemond-Hernandez *et al.*, 2010), the highest number of resistant isolates were against benzylpenicillin, erythromycin, and oxacillin. The resistance against these antibiotics in the current finding is similar to earlier reports (Lebowitz *et al.*, 2017; Neyra *et al.*, 2014). Vigilant use of the latest generation antibiotics is mandatory as a significant number of resistant isolates against ciprofloxacin, clindamycin, and erythromycin were identified in this study. Resistance against those latest generation antibiotics are available in the literature (Hiramatsu *et al.*, 2014; Sekhar *et al.*, 2014). The repeated antimicrobial treatment may partly explain the overall high rates of MDR *S. aureus* and MRSA in DFU patients due to the chronic and polymicrobial nature of the infection (Bertesteanu *et al.*, 2014; Trivedi *et al.*, 2014; Dalton *et al.*, 2011; Demling and Waterhouse, 2007).

The increase in the prevalence of antibiotic-resistant pathogenic bacteria, including *S. aureus,* has led to an increased search for alternative therapeutic agents with low cost, easy availability, minimum side effects, and high efficacy. Phages may fulfill some of these features and kill bacterial hosts irrespective of their antibiotic susceptibility (Kelly *et al.*, 2011). Hence, phages are increasingly becoming a focus of research (Chan *et al.*, 2013; O'Flaherty *et al.*, 2009). This study shows that at least 75% and 85% of *S. aureus* isolates are susceptible to each component phage and phage cocktail AB-SA01, respectively. Similar efficacy and host range were reported for Sa 83, and Sa 87 phages on antibiotic-resistant and

antibiotic-susceptible *S. aureus* isolates collected from chronic rhinosinusitis patients (Zhang *et al.*, 2018).

Although it is usually envisaged that phage cocktails are better effective (synergy) than single phage treatment to a particular bacterial host (Schmerer *et al.*, 2014), there is a rare possibility that coinfection of component phages of the phage cocktail to a bacterium negatively affect the outcome of phage infection (Abedon, 1990). There may be repeated secondary phage adsorptions resulting in the continuous induction of lysis inhibition (Abedon, 1992). This lysis inhibition occurs when the second phage adsorption extends the latent period of the first phage (Cairns and Payne, 2008; Abedon, 1992; Abedon, 1990). This phenomenon could be suggested as one of the reasons for the higher host range observed in one of the phage components than the phage cocktail used in this study. As a limitation to this study, specific receptors were not identified for each component phage of AB-SA01.

In the present study, the phage cocktail AB-SA01 produced a better quality of lysis (clear and full lysis) than any of its components. Clear and full lysis by the phage cocktail was observed on more than 95% of the susceptible isolates, including those resistant to or partially lysed by the component phages. The better efficacy of the phage cocktail shows that another component phage could lyse resistant isolate to one component phage in the mixture (Lehman *et al.*, 2019). The use of a phage mixture is mostly preferred over single phage as it results in a decreased rate of resistant mutant development (Gu *et al.*, 2012; Tanji *et al.*, 2005). The current findings were consistent with previous studies on the efficacy of various phage cocktails and component phages of *S. aureus* (Leskinen *et al.*, 2017; Mendes *et al.*, 2014; Kelly *et al.*, 2011). The findings of this study indicate that AB-SA01 and its components are effective on antibiotic-resistant and antibiotic-susceptible *S. aureus* isolates. AB-SA01 is likely safe to treat *S. aureus* infections as it complied with the U.S. Food and

Drug Administration (FDA) and Australia's Therapeutic Goods Administration (TGA) requirements to conduct clinical trials and single-patient emergency treatment (Lehman *et al.*, 2019).

Most of the *S. aureus* isolates used in this study produced low-grade biofilm. This finding agrees with reports on *S. aureus* strains isolated from various sources (Avila-Novoa *et al.*, 2018; Szczuka *et al.*, 2013; Tang *et al.*, 2013; Gutierrez *et al.*, 2012). The present study result also lends strong support to the understanding that 99% of microbes present in ecosystems may grow as biofilms (Parasion *et al.*, 2014). The high rate of MRSA, MDR *S. aureus*, and MDR MRSA is also consistent with the fact that the ability to form biofilm increases resistance to antibiotics (Cervantes-Garcia *et al.*, 2015; Percival *et al.*, 2012).

S. aureus is known for producing biofilm in wounds that may be one cause of persistent and relapsing antibiotic-resistant infections in DFU patients (Shettigar *et al.*, 2016; Cervantes-Garcia *et al.*, 2015; Bjarnsholt *et al.*, 2008). Limited studies suggest the significant potential of phages to reduce or eliminate biofilms (Drilling *et al.*, 2014; Alves *et al.*, 2014; Kelly *et al.*, 2012; Cerca *et al.*, 2007). AB-SA01 showed a significant decrease in the biofilm biomass on the laboratory strains and clinical isolates of *S. aureus.* All isolates that were resistant and intermediately susceptible to AB-SA01 in the planktonic assays showed little (< 10%) to no biofilm biomass reduction. Biofilms developed from five *S. aureus* isolates, which were strongly susceptible to AB-SA01 in spot test, also did not show significant biomass reduction. The insignificant biofilm biomass reduction in about 20% of the isolates during phage cocktail treatment in this study was not unexpected. It is because bacteria in the biofilm state are more resistant to phage attacks than their counterparts in the planktonic phase (Fu *et al.*, 2010).

Studies have shown the promising efficacy of phage mixtures on the reduction of biofilm biomass of nonhuman and human clinical isolates of *S. aureus* (Alves *et al.*, 2014; Kelly *et al.*, 2011). Here, the biofilm biomass reduction capability of phage cocktail AB-SA01 on biofilms produced by *S. aureus* isolates collected from DFU patients was examined. This study suggests that AB-SA01, a phage cocktail that has undergone two Phase I trials and met the cGMP standard (Lehman *et al.*, 2019), therapy could provide a practical alternative to treat multidrug-resistant and biofilm-associated *S. aureus* infections. The present study shows the effectiveness of a phage cocktail on MDR MRSA and MDR *S. aureus* clinical isolates collected from DFU patients and their biofilms.

After trialing disinfectants (triton x-100, 2.5% cetylpyridinium chloride (CPC)) and tetracycline as a positive control (data provided under appendices), tetracycline 16 µg/ml was found to be better in reducing biomass on 48 hours biofilms than the disinfectants tested. Besides, tetracycline was selected as a positive control since it was effective on both *S. aureus* and *P. aeruginosa* isolates selected for mixed-species planktonic and biofilm cultures treatment experiments (discussed in chapter 4). Hence, tetracycline was found to be an appropriate positive control that substantially decreased the biofilm biomass in most isolates tested. There was no statistically significant difference in biofilm biomass reduction ($p < 0.05$) between AB-SA01 and tetracycline treatment. In this study, a higher concentration of tetracycline (16 µg/ml) was used because the MIC for all isolates obtained during the VITEK® 2 test was < 8 µg/ml. The reasons for biofilm's resistance to disinfectants and antibiotics are usually multifaceted but mainly due to intrinsic resistance mechanisms and adaptive stress responses (Stewart, 2002).

Some reports indicated that biofilms have water-filled tubules that facilitate phage movement within the biofilms (Donlan, 2009; Sutherland *et al.*, 2004; Doolittle *et al.*, 1996). However,

other reports suggested that the presence of bacterial proteolytic enzymes that can inactivate phages, and the dormant metabolic state of the bacterial cells in the biofilm could hamper phage lytic activity (Gutierrez *et al.*, 2015; Cerca *et al.*, 2007; Doolittle *et al.*, 1996). These deterring factors could be the possible explanations for the lesser efficacy of AB-SA01 in the biofilm than planktonic states in this study.

In summary, this study describes the efficacy of phage cocktail AB-SA01 and its phage constituents against pathogenic and antibiotic-resistant *S. aureus* from DFU patients. Each phage and their combination showed a broad host range of infectivity on *S. aureus* isolates. Furthermore, the phage cocktail demonstrated efficacy in biofilm biomass reduction superior or similar to tetracycline, suggesting AB-SA01 is the right candidate for further development to treat chronic wound infections caused by *S. aureus*. There was no statistically significant difference in phage sensitivity between antibiotic-resistant and antibiotic-sensitive *S. aureus* clinical isolates. The strong lytic efficacy of phages, including on MDR *S. aureus* isolates, shows the promising prospect of AB-SA01 and its components as alternatives to antibiotics.

Chapter 3

Effect of Phage Cocktail AB-PA01 on *Pseudomonas aeruginosa* Diabetic Foot Ulcer Clinical Isolates and Biofilms

Chapter 3: Effect of Phage Cocktail AB-PA01 on *Pseudomonas aeruginosa* Diabetic Foot Ulcer Clinical Isolates and Biofilms

Abstract

Pseudomonas aeruginosa infections are common amongst diabetic foot ulcer (DFU) patients. Most *P. aeruginosa* strains are antibiotic-resistant and form a biofilm, both of which may lead to treatment failure. This study describes the effect of a cGMP-quality phage cocktail AB-PA01 and four of its components on *P. aeruginosa* isolates from DFU infection patients, with phage applied to bacteria in planktonic and biofilm states. The objective of this study was to assess the activity of the four *P. aeruginosa* phages and their mixture, AB-PA01, against *P. aeruginosa* cells and biofilms *in vitro.* Bacterial isolates grown in the planktonic state were treated with AB-PA01 and its components and their biofilms with AB-PA01. The effect of phages on planktonic cells was assessed using a spot test. Biofilm biomass was measured using a crystal violet (CV) assay. Of 41 isolates tested, nearly 93% were strongly susceptible to AB-PA01. Moreover, 66% – 88% of isolates were susceptible to each of the four phages. AB-PA01 treatment significantly reduced biofilm biomass ($p < 0.001$), regardless of the antibiotic-resistant characteristics of the isolates. Furthermore, a single dose of a phage cocktail is capable of significantly reducing biofilms formed *in vitro* by a range of *P. aeruginosa* isolates from DFU patients. The lytic efficacy and broad host range of AB-PA01 and its components suggest the enormous potential of phages in the treatment of *P. aeruginosa* infections.

3.1 Introduction

3.1.1 *P. aeruginosa* in DFUs

DFUs cause substantial morbidity, impaired quality of life, and prolonged antibiotic treatment and hospitalization; they may require surgical interventions, and impose high health care cost (Wu *et al.*, 2017; Petrakis *et al.*, 2017; Lipsky *et al.*, 2016; Lipsky *et al.*, 2012a; Lavery *et al.*, 2006; Lavery *et al.*, 2003). Nearly 25% of people living with diabetes will be affected by DFUs in their lifetime, and 80% of diabetic-related lower-extremity amputations are due to DFUs (Hingorani *et al.*, 2016; Boulton *et al.*, 2005). Most DFU cases become infected (Lavery *et al.*, 2003). Often, DFU infections are polymicrobial (Mottola *et al.*, 2016; Dowd *et al.*, 2008) and associated with MDR microorganisms (Murali *et al.*, 2014). The common causative bacteria in DFUs differ by geographical regions; for instance, *P. aeruginosa* is common in warm and humid climates found in parts of Asia and Africa (Wu *et al.*, 2017; Hatipoglu *et al.*, 2014).

P. aeruginosa is a common pathogen in DFU infections, which associates with prolonged duration of antibiotic therapy and poorer outcomes (Commons *et al.*, 2015; Illgner *et al.*, 2013; Lipsky *et al.*, 2010). *P. aeruginosa* can establish an active and persistent infection in diabetic wounds (Goldufsky *et al.*, 2015). It often resides deep in tissues and is protected from antibiotics and the immune system due to biofilm formation. Another reason for the seriousness of DFU infections with *P. aeruginosa* is the ability of the bacteria to express virulence factors such as flagella, pili, and lipopolysaccharide. Moreover, toxin production and quorum sensing of *P. aeruginosa* significantly contribute to its pathogenesis and poor treatment outcome in DFUs (Ertugrul *et al.*, 2018; Høgsberg *et al.*, 2011). These factors lead to higher rates of unsuccessful treatment and often to lower extremity amputations (Petrakis *et al.*, 2017). DFU infections impose a significant health and financial problem on patients

and substantial economic pressure on health care institutions all over the globe (Petrakis *et al.*, 2017).

3.1.2 Identification of *P. aeruginosa*

P. aeruginosa can be challenging to identify using routine techniques as it is inert for some biochemical activities and difficult to interpret some of the phenotypic characteristics (Kacaniova *et al.*, 2019; Marko *et al.*, 2012). MALDI-TOF MS, a simple, cost-effective, rapid, discriminatory, and reliable technique, effectively identifies *P. aeruginosa* colonies with high sensitivity and specificity (Kacaniova *et al.*, 2019; Gautam *et al.*, 2017; Barnini *et al.*, 2015; Marko *et al.*, 2012). MALDI-TOF MS is used for species identification in many diagnostic microbiological laboratories (Angeletti, 2017; Schaumann *et al.*, 2012). It uses soft laser ionization to detect peptide and protein ions based on their relative masses and charges for identification since each bacterium has different spectra based on their mass charge ratio (Harris *et al.*, 2010; Bernardo *et al.*, 2002; Edwards-Jones *et al.*, 2000).

MALDI-TOF MS can detect and distinguish hundreds of proteins and peptides in seconds through a comparison of the spectra with those in a reference database (Angeletti, 2017; Barreiro *et al.*, 2017; Gagnaire *et al.*, 2012). In most cases, a mass-to-charge ratio of ≥ 2.00 is recommended for species-level identification (Camoez *et al.*, 2016; Elbehiry *et al.*, 2016; Harris *et al.*, 2010). These principles were employed to identify *P. aeruginosa* clinical isolates collected from DFU patients who visited various hospitals in the Adelaide area during the study period.

3.1.3 Antimicrobial susceptibility of *P. aeruginosa*

Antibiotic therapy with proper wound dressing, including surgical intervention, where appropriate, is the primary treatment option for DFU infection (Ertugrul *et al.*, 2017). Prompt

identification of causative agents of DFU infection could limit indiscriminate use of broad-spectrum antibiotics and improves antibiotic stewardship (Lebowitz *et al.*, 2017). *P. aeruginosa* has become resistant to many commonly prescribed antibiotics due to resistance mechanisms, including efflux pumps, antibiotic degrading enzymes, low membrane permeability, and strong biofilm formation capability (Mah *et al.*, 2003; Drenkard and Ausubel, 2002; Stewart, 2002). Broad-spectrum antibiotic treatment carries a risk of selection of resistant microorganisms and affecting normal flora of the skin, hence selected antibiotics with known efficacy on the specified pathogen should be used (Barwell *et al.*, 2017).

Better treatment outcomes are often associated with timely and appropriate antibiotic treatment based on the identified pathogen susceptibility pattern (Wu *et al.*, 2017). The sensitivity and specificity of antimicrobial susceptibility tests for *P. aeruginosa* have a long-standing concern and better explained through inter-methods comparisons of automated systems (Pfaller *et al.*, 2014). Reports showed that VITEK® 2 performance is satisfactory for antimicrobial susceptibility testing of Gram-negative bacteria, including *P. aeruginosa* (Bobenchik *et al.*, 2017; Juretschko *et al.*, 2007). VITEK® 2 comprises a database of MICs for various antibiotics and the prevalent resistance mechanisms in different species, together with a series of algorithms (Bobenchik *et al.*, 2015; Lavallee *et al.*, 2010; Saegeman *et al.*, 2005). The VITEK® 2 system automatically measures a turbidity signal for each test well containing an antibiotic, every 15 minutes for up to 18 hours. These data are used to generate growth curves, and MIC of each antibiotic is estimated by comparison with a control (Saegeman *et al.*, 2005; Sanders *et al.*, 2000; Doern *et al.*, 1997). All *P. aeruginosa* species examined in the current study were evaluated for antibiotic susceptibility profile using VITEK® 2.

3.1.4 Phages as an alternative treatment for *P. aeruginosa*

The growing evidence of an increasing number of MDR *P. aeruginosa* strains has prompted the search for new therapeutic agents. Potential alternatives are lytic phages, which have the self-replicating ability, high specificity, and abundance in the environment (El Didamony *et al.*, 2015; Sulakvelidze, 2013). Phages are potentially effective against MDR bacterial infections because the mechanism by which they produce their lytic effect is not related to antibiotic resistance mechanisms (Shivshetty *et al.*, 2014). Phages can reportedly lyse their host bacteria even when inside biofilms (Holguin *et al.*, 2015; Krylov *et al.*, 2013; Kumari *et al.*, 2009; McVay *et al.*, 2007). Information about phage susceptibility of chronic DFU infections causing *P. aeruginosa* isolates and availability of effective phages is limited.

Exclusively lytic *P. aeruginosa* phages are diverse and distributed in at least seven genera in which different host ranges are observed (Essoh *et al.*, 2013). Narrow host range and emergence of resistant host strains are frequently listed among the shortcomings of phage therapy (Kwiatek *et al.*, 2015). Phage cocktail application is recommended to alleviate phage resistant problem and broaden the host range (Bernasconi *et al.*, 2017; Alves *et al.*, 2016; Schmerer *et al.*, 2014; Chan *et al.*, 2013; Gu *et al.*, 2012; Kelly *et al.*, 2011).

3.1.5 Phage cocktail to treat *P. aeruginosa* infections

It is possible to broaden the host range of phage treatment by combining various lytic phage types possessing different host ranges. Hence, phage cocktails may be more effective than single phage to treat bacterial diseases (Alves *et al.*, 2016; Born *et al.*, 2011). Furthermore, phages in the cocktail should be chosen to complement each other so that resistant mutants to one phage are susceptible to another (Lehman *et al.*, 2019; Mendes *et al.*, 2014). There are reports of effective therapeutic phage cocktail formulations to treat diseases ranging from

skin wound to systemic infections (Manohar *et al.*, 2019; Seo *et al.*, 2018; Ramirez *et al.*, 2018; Schooley *et al.*, 2017; Leskinen *et al.*, 2017; Bernasconi *et al.*, 2017; Gundogdu *et al.*, 2016; Alves *et al.*, 2016; Abdulamir *et al.*, 2015; Mendes *et al.*, 2014; Hooton *et al.*, 2011; Fu *et al.*, 2010; Merabishvili *et al.*, 2009; O'Flynn *et al.*, 2004). The phage cocktail, AB-PA01, evaluated in this study, is a constant-composition of naturally occurring and obligately lytic two *Myoviridae* and two *Podoviridae*. AB-PA01 is produced to treat *P. aeruginosa* infections, and none of its components has any known bacterial virulence or antibiotic resistance genes (Fong *et al.*, 2017).

3.2 Materials and methods

3.2.1 Identification of *P. aeruginosa* isolates

P. aeruginosa clinical isolates were collected from infected DFU patients from diabetic foot clinics in Adelaide, South Australia, during the study period and cultured on Muller Hinton agar. Single colonies of the presumed *P. aeruginosa* isolates from Muller Hinton agar plates that were generously provided by South Australia Pathology were subcultured onto vancomycin-supplemented MacConkey agar (Thermo Fisher, South Australia) following the established protocol (Eckmanns *et al.*, 2008; Kirisits *et al.*, 2005; Wuthiekanun *et al.*, 1990; Brodsky and Nixon, 1973). After 24 hours of incubation at 37 ^{o}C, MacConkey plate cultures were stored under 4 ^{o}C until further analysis. *P. aeruginosa* laboratory strains ATCC 15692 (PAO1) and ATCC 10145, generously provided by Dr. Nicky Thomas from the University of South Australia and Professor Melissa Brown from Flinders University, respectively, were used as quality control references.

A colony of each *P. aeruginosa* isolates showing typical characteristics on 18 hours vancomycin-supplemented MacConkey agar culture was applied to a MALDI-TOF MS sample plate using a sterile inoculation stick. Vancomycin was applied on MacConkey agar as a supplement to hinder Gram-positive bacteria growth. Vancomycin is one of the commonly prescribed antibiotics to treat most Gram-positive bacterial infections and was effective on all *S. aureus* isolates tested in this study. One microliter of 70% formic acid and 1 µl of the alpha-cyano-4-hydroxycinnamic acid (HCCA) (Sigma-Aldrich Pty Ltd, New South Wells, Australia) matrix was applied following the manufacturer's instructions. Identification of the bacterial species was performed and analyzed using Bruker Daltonik MALDI Biotyper 3.0 (Bruker Pyt Ltd, Victoria, Australia), as previously described

(Kacaniova *et al.*, 2019; Hoyos-Mallecot *et al.*, 2014). The isolates were considered *P. aeruginosa* when the scores are ≥ 2.00 as both first- and the second-best match organism. After identification, all isolates were stored in 25% glycerol nutrient broth at -150 ^{o}C until further analysis.

3.2.2 Antibiotic susceptibility profile of *P. aeruginosa* isolates

P. aeruginosa isolates identified as above by MALDI-TOF MS were cultured on vancomycin-supplemented MacConkey agar for 18 - 24 hours at 37 °C aerobically. Three colonies were mixed with 3 ml of 0.45% saline and adjusted to the turbidity of a 0.5 - 0.63 McFarland standard following the manufacturer's instruction. Then, 145 µl of the bacterial suspension was mixed with another 3 ml of 0.45% saline solution and loaded with antimicrobial susceptibility test cards and incubated in the VITEK® 2 machine (bioMérieux Australia Pty Ltd, New South Wales, Australia) for overnight analysis as described earlier (Juretschko *et al.*, 2007; Sader *et al.*, 2006; Bruins *et al.*, 2004; Sanders *et al.*, 2000; Doern *et al.*, 1997). A loopful of the suspension was also cultured on blood agar overnight at 37 ^{o}C to check the presence or absence of contamination during sample handling. The susceptibility test card (VITEK® 2 AST-N246) contains the following antibiotics: ticarcillin/clavulanic acid, piperacillin/tazobactam, ceftazidime, cefepime, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, and norfloxacin.

3.2.3 Phage titer

Phage cocktail AB-PA01 and its components Pa 193, Pa 204, Pa 222, and Pa 223 donated by AmpliPhi Biosciences Corporation were used in this study. Pa 193 and Pa 204 are *Myoviridae,* and Pa 222 and Pa 223 are *Podoviridae*. The titer of the phage cocktail and component phages was determined using double agar overlay plaque assay, as indicated

previously (Mirzaei and Nilsson, 2015; Merabishvili *et al.*, 2009) on known susceptible *P. aeruginosa* laboratory strains PAO1 and PA10145 and two clinical isolates.

Dilution of phage solutions up to 10^{-8} dilution was performed in TSB after filter sterilization. One-hundred microliters of each phage and phage cocktail solution from 10^{-4} - 10^{-8} dilutions and 100 µl of 18 hours broth culture of each strain or isolate were mixed with 3 ml trypticase soy soft agar maintained at 42 ^{O}C, uniformly dispensed over pre-warmed at 37 ^{O}C TSA plates, and incubated overnight at 37 °C. Plaques were counted on the following day, and the phage titer was expressed as log-transformed plaque-forming units per milliliter (PFU/ml).

Each phage and phage cocktail solution was tested three times for each dilution. PFU counting was conducted on plates estimated to contain 30 - 300 distinguishable homogenous PFU from each dilution. The average number of PFU was taken and calculated by the following equation to estimate the original stock phage solution titer, $pfu/ml = \frac{Number\ of\ plaques}{d \cdot V}$, where *d* stands for dilution factor and *V* for a volume of inoculum, the mean was then calculated for the three plates.

3.2.4 Phage efficacy and host range test

Evaluation of lytic efficacy of phage cocktail AB-PA01 and its components using a spot test was performed based on the established procedure (Mirzaei and Nilsson, 2015; Alves *et al.*, 2014). Briefly, 100 µl of 16 - 18 hours culture of *P. aeruginosa* isolates was mixed with 3 ml of trypticase soy soft agar and poured onto pre-warmed at 37 ^{O}C TSA plates. After ensuring the even distribution of the soft agar suspension, plates were left to dry for 30 minutes at room temperature. Ten microliters of each phage and phage cocktail solutions adjusted to 9.0 $\log_{10}$ (PFU/ml) titer were serially diluted with TSB, and 10 µl of 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7}

dilutions were spotted onto the bacterial lawns in triplicate. Ten microliters of sterile PBS were used as a positive control.

The plates were kept at room temperature for 30 minutes and incubated inverted at 37 ^{o}C overnight. This assay was performed in triplicate. The susceptibility patterns of the isolates were examined the following day. The observation was recorded according to AmpliPhi Biosciences Corporations' recommendation: (i) resistant if very weak to no activity is seen, (ii) intermediate when faint and turbid spots or partial activity is observed, and (iii) susceptible when a clear spot or full activity with no bacterial growth is observed within the spots. The results taken as final were the mean of the triplicate tests.

3.2.5 Effect of AB-PA01 on *P. aeruginosa* biofilms

The biofilm development on a flat-bottom transparent CELLSTAR polystyrene 96-well microplate (Sigma-Aldrich, NSW, Australia) was carried out as detailed earlier (Alves *et al.*, 2016; Pires *et al.*, 2011; Spoering and Lewis, 2001). *P. aeruginosa* isolates were cultured overnight on vancomycin-supplemented MacConkey agar under the aerobic condition at 37 ^{o}C to yield isolated colonies. After 18 hours of incubation, a single colony was transferred into a sterile glass test tube of 0.45% saline and adjusted to 1.0 McFarland turbidity standard.

The bacterial suspension in saline was diluted by a 1:100 ratio in 1% glucose Luria-Bertani (LB) broth, and 150 µl of the suspension was transferred to the sterile wells of a microplate. After 48 hours of incubation at 37 ^{o}C with agitation by a gyratory mixer at 70 rpm, the liquid part of the culture was removed through gentle pipetting. The plates were washed with sterile deionized water three times by careful pipetting not to disrupt the biofilm. Plates were left at room temperature in a biosafety cabinet to dry in the air, and then 180 µl phage-, gentamicin-

(positive control), tetracycline- (positive control), and sterile PBS-broth (negative control) solutions were applied.

The treatment-broth solutions were constituted: (i) 18 µl phage solution (at 9 log_{10} (PFU/ml)) mixed with 162 µl LB broth, (ii) 23 µg tetracycline in 180 µl LB broth, (iii) 16 µg gentamicin in 180 µl broth, and (iv) 18 µl PBS in 162 µl LB broth. The concentration of tetracycline was 128 µg/ml because *P. aeruginosa* isolates are susceptible for a higher concentration of tetracycline (Zheng *et al.*, 2017; Lister *et al.*, 2009; Pai *et al.*, 2001; Li *et al.*, 1994; Petersen *et al.*, 1999). The higher MIC of gentamicin than from VITEK® 2 test results was used for biofilm treatment.

The treated biofilms were incubated at 37 ^{o}C under aerobic and static conditions. After 12 hours of incubation, the fluid portion of the culture was gently removed, biofilms were washed twice through gentle immersing of the whole microplate into distilled water and dry the microplates gently onto a paper towel. Then, the biofilms were fixed using 95% methanol for 30 minutes. Methanol was decanted, and plates were washed once with sterile distilled water and air-dried. The fixed biofilms were then stained with 190 µl per well of 0.2% CV. After staining for 60 minutes, excess CV stain was removed by gentle washing twice using distilled water and left in a dark room overnight to dry. The stained biofilm was eluted at room temperature for 30 minutes using 200 µl of 30% acetic acid.

The eluted suspension was transferred into a new microplate, and the optical density of the suspension was measured at 600 nanometers (600_{nm}) after including 30% acetic acid as a negative control. A FLUOstar® Omega multi-mode microplate reader (BMG LABTECH Pty. Ltd., Victoria, Australia) was employed to read the optical density following the manufacturer's instruction. The OD reading data was analyzed and compared with the blank-corrected mean of the three replicates. The percentage of biofilm biomass reduction (%BK)

was calculated from the absorbance of background-corrected untreated controls (I_C, PBS treated) and the absorbance of the treatments (I_T) (Thomas *et al.*, 2015) as follows:

$$\%BK = \frac{I_C - I_T}{I_C} \times 100\%$$

Based on the biofilm biomass they produced, the isolates were categorized as (i) non-biofilm producer if $OD_{600} < 0.1$, (ii) weak biofilm producer when $0.1 \leq OD_{600} < 1$, and (iii) strong biofilm producer if $OD_{600} \geq 1$ based on literature (Avila-Novoa *et al.*, 2018; Rohde *et al.*, 2007). The biofilm biomass reduction as a result of treatment effect was categorized: (i) insignificant ≤ 20%, (ii) weak 20.1- 40.0%, (iii) moderate 40.1- 60.0%, (iv) strong 60.1- 80.0 %, and (v) very strong ≥ 80% reduction.

3.2.6 Data management and statistical analysis

Microsoft Excel Spreadsheet was used to double enter, encode, and store the data. STATA version 16 software was used for statistical analysis. Data are reported as the mean ± standard error of the mean and are compiled from at least three separate experiments and are expressed as logarithm-transformed values $\log_{10}$ (CFU /ml) over time. A comparison of experimental groups was performed using a one-way analysis of variance (two-tailed) or paired 't-test'. A statistical significance was considered at $p < 0.05$.

3.3 Results

3.3.1 Isolation of *P. aeruginosa* using culture

Swab samples from wounds of DFU patients who visited various diabetic clinics in Adelaide were collected and cultured aerobically at 37 ^{o}C onto Muller Hinton agar plates by South Australia Pathology. Colonies presumed to be *P. aeruginosa* isolates were subcultured onto another Muller Hinton agar and sent to our laboratory for further identification, antimicrobial susceptibility, and phage treatment experiments. Mucoid and pigmented or sometimes metallic sheen, sticky (hyper-adherent), and aggregative colonies on Muller Hinton agar were considered *P. aeruginosa* isolates. Isolated colonies were taken and plated on 1.2% MacConkey agars supplemented with vancomycin to suppress the growth of Gram-positive bacteria. Based on colony characteristics on vancomycin-supplemented MacConkey agar, 44 *P. aeruginosa* clinical isolates were isolated. These were stored in 25% glycerol nutrient broth at -150 ^{o}C until further analysis.

3.3.2 Identification of *P. aeruginosa* using MALDI-TOF MS

All presumed *P. aeruginosa* isolates were typed using MALDI-TOF MS, and results were analyzed with Biotyper 3.0. Isolates with $\geq$ 2.00 score for both the first- and second-best matches were identified as *P. aeruginosa.* Of 44 clinical isolates tested, 88.6% (n = 39) were confirmed *P. aeruginosa.* The remaining isolates were *Proteus mirabilis* (2), *Serratia marcescens* (1), *Enterococcus faecalis* (1), and *Klebsiella pneumonia* (1). Reference *P. aeruginosa* strains PAO1 and PA10145 were also confirmed with good scores. All confirmed isolates were tested for antibiotic susceptibility against commonly prescribed drugs using the VITEK® 2 system.

3.3.3 Antimicrobial susceptibility of *P. aeruginosa* using VITEK® 2

All 41 *P. aeruginosa* isolates were susceptible to meropenem, amikacin, and tobramycin, as shown in Figure 3.1. Nearly 98% (n = 40) and 95% (n = 39) isolates were also susceptible to norfloxacin and gentamicin, respectively. The highest proportion of resistant isolates, 48.8% (n = 20), was found against ticarcillin/clavulanic acid. The next higher number of resistant isolates, 14.6% (n = 6), was observed against piperacillin/tazobactam. One isolate was resistant to half of the antibiotics (n = 5) it was exposed to and was considered MDR.

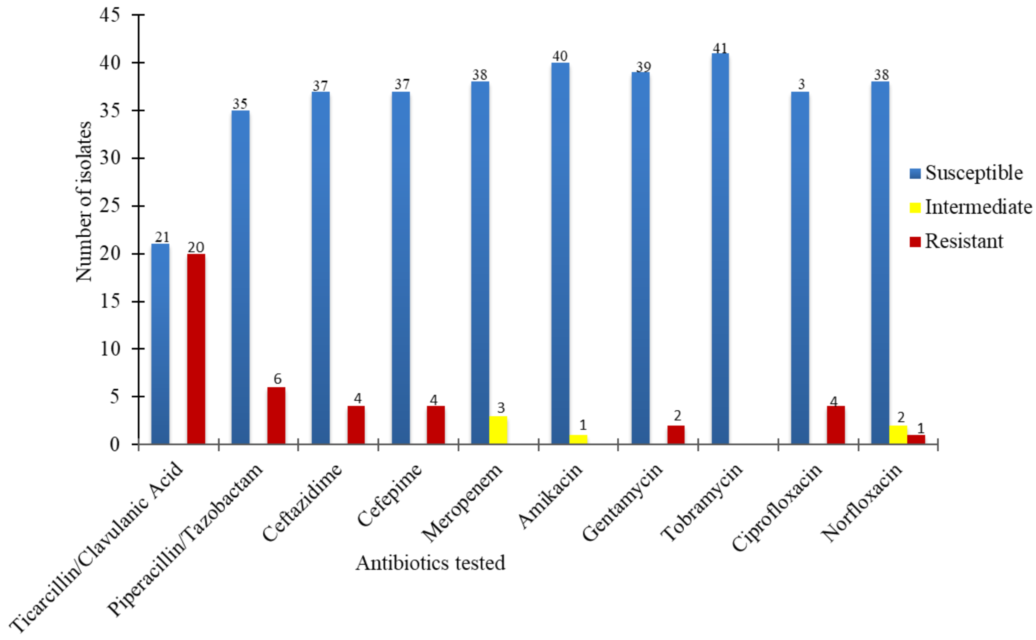

Figure 3. 1 Antibiotic susceptibility of *P. aeruginosa* isolates collected from DFU patients.

3.3.4 Titer of the phage cocktail AB-PA01 and its components

Plates with clear isolated plaques, as shown in Figure 3.2, were selected for each phage and phage cocktail PFU count. The finding was 10.4 – 10.5 $\log_{10}$ (PFU/ml) mean titer for Pa 193, Pa 204, and Pa 222, and 9.5 $\log_{10}$ (PFU/ml) mean titer for Pa 223. The mean titer of the phage cocktail AB-PA01, the combination of these four phages, was 10.3 $\log_{10}$ (PFU/ml).

Consistent phage titers were obtained for each phage and the phage cocktail on the two clinical isolates and two laboratory strains.

Figure 3. 2 PFU of AB-PA01 on *P. aeruginosa* PA01 during evaluation phage titer.

3.3.5 Lytic efficacy and host range of phages on spot test

P. aeruginosa isolates were considered susceptible to phages when partial or full activity was observed on the spot test, as shown in Figure 3.3. Of the 41 isolates tested, 87.8% (n = 36), 73.1% (n = 30), and 65.9% (n = 27) were lysed by Pa 193 and Pa 222, Pa 204, and Pa 223, respectively, as illustrated in Figure 3.4. Five percent (n = 2) isolates were resistant against all component phages separately tested in this study. One isolate was resistant to all phages and the phage cocktail.

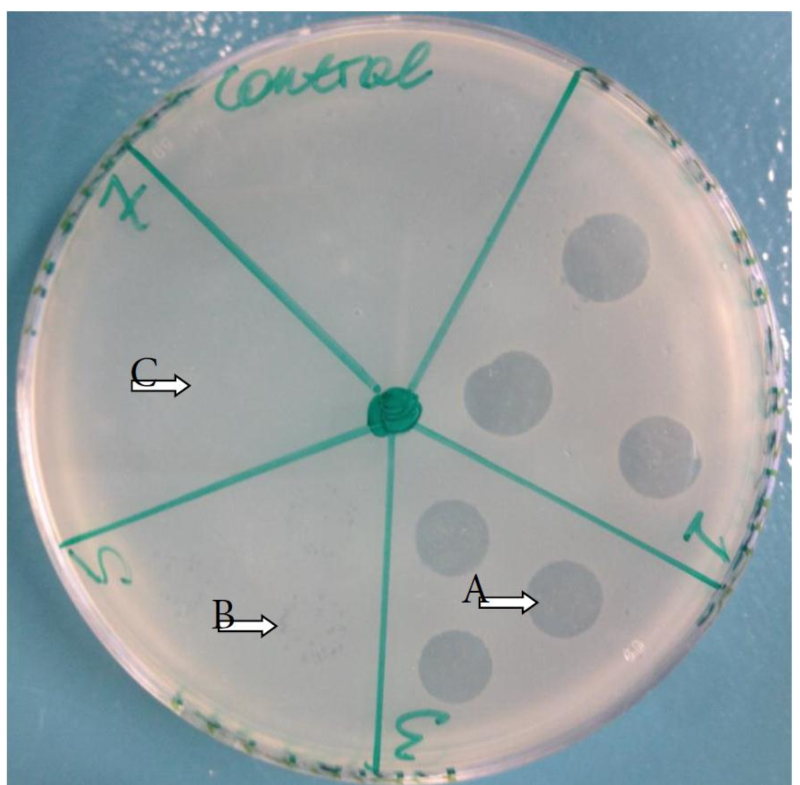

Figure 3. 3 Lytic activity of AB-PA01 on *P. aeruginosa* isolates: **A** complete lysis, **B** intermediate lysis, and **C** no lysis.

The phage cocktail, AB-PA01, lysed 92.7% (n = 38) of the isolates, and 89.5% (n = 34) of the lysis was complete, which was large and clear plaque without visible colony inside the spotted area. Isolates resistant against AB-PA01 were also resistant to at least two of the component phages. One *P. aeruginosa* isolate, which was MDR during the VITEK® 2 test, was susceptible to all component phages and the phage cocktail. In most of the cases, isolates that showed resistance to one phage also showed resistance to at least one other phage tested. Two *P. aeruginosa* isolates were resistant to both *Myoviridae* phages, and the other two isolates were resistant to *Podoviridae* phages only.

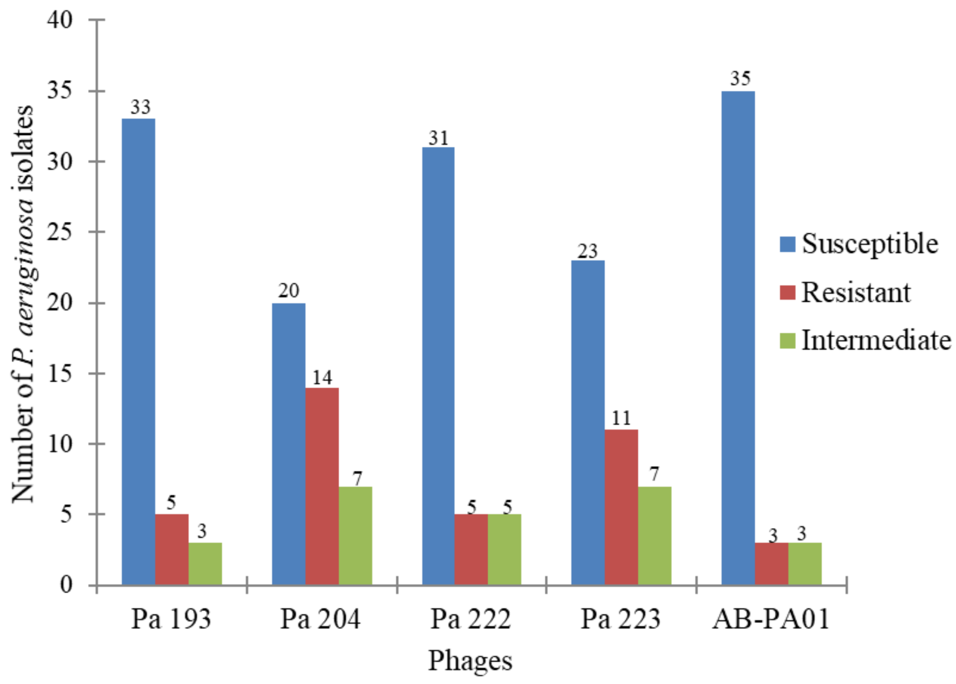

Figure 3. 4 Phage susceptibility of *P. aeruginosa* isolates collected from DFU patients.

3.3.6 Effect of AB-PA01 on biofilms of *P. aeruginosa* isolates

Of the 41 *P. aeruginosa* isolates cultured for biofilm development on 96-well as described in the material and method section, 97.6% (n = 40) isolates produced biofilm. The OD reading of the biofilm at 600_{nm} ranged from 0.17 to 3.14. Accordingly, more than 73% (n = 30) of the isolates were strong biofilm producers, as shown in Figure 3.5.

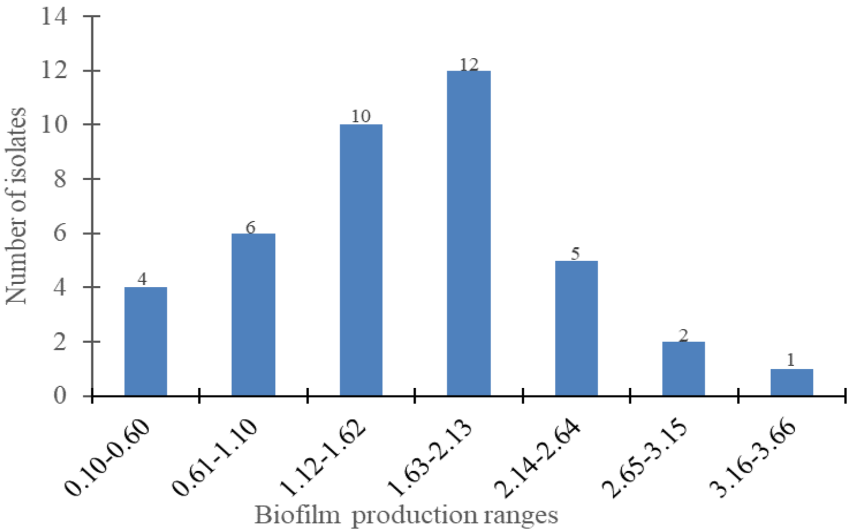

Figure 3. 5 Biofilm formation capability of *P. aeruginosa* isolates from DFU patients.

After treating the 48 hours biofilms with AB-PA01 at MOI 1 for 12 hours and staining with CV, the biofilm biomass was measured using the FLUOstar® spectrophotometer at OD_{600nm}. The biofilm biomass reduction due to AB-PA01 treatment was compared with PBS treatment. Tetracycline and gentamicin were used as positive controls. Based on the criteria described above, 17.5% (n = 7), 22.5% (n = 9), 30% (n = 12), and 12.5% (n = 5) of the isolates showed weak, moderate, strong, and very strong biofilm biomass reduction, respectively, due to AB-PA01 treatment effect as shown in Figure 3.6.

In summary, 82.5% of isolates biofilms showed significant biomass reduction because of AB-PA01 treatment. Of the remaining 17.5% (n = 7) isolates, four isolates did not show significant biofilm biomass reduction (< 20%), whereas the other three showed 10.6 - 26.3% biofilm biomass increase after phage cocktail treatment. The maximum biomass reduction observed in this study was 88.2%. Overall, statistically significant ($p < 0.001$; 0.82 vs 1.60) biofilm biomass reduction was observed because of AB-PA01 treatment compared to the negative treatment (PBS) control group.

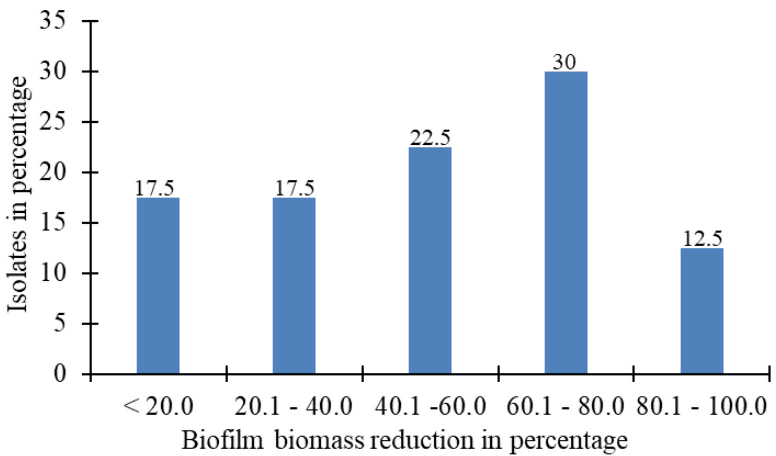

Figure 3. 6 Effect of AB-PA01 on *P. aeruginosa* isolates biofilm biomass reduction.

There was no statistically significant difference ($p > 0.05$; 0.60 vs 0.58) between AB-PA01 and tetracycline treated group in biofilm biomass reduction, as shown in Figure 3.7. The OD_{600nm} reading of AB-PA01- and tetracycline-treated biofilms were substantially reduced

compared with PBS treated (negative control) biofilms. Generally, the biofilm biomass reduction because of phage cocktail AB-PA01 and tetracycline treatment was strongly statistically significant ($p < 0.001$; 0.21 vs 0.81 and 0.23 vs 0.81) compared with the negative control.

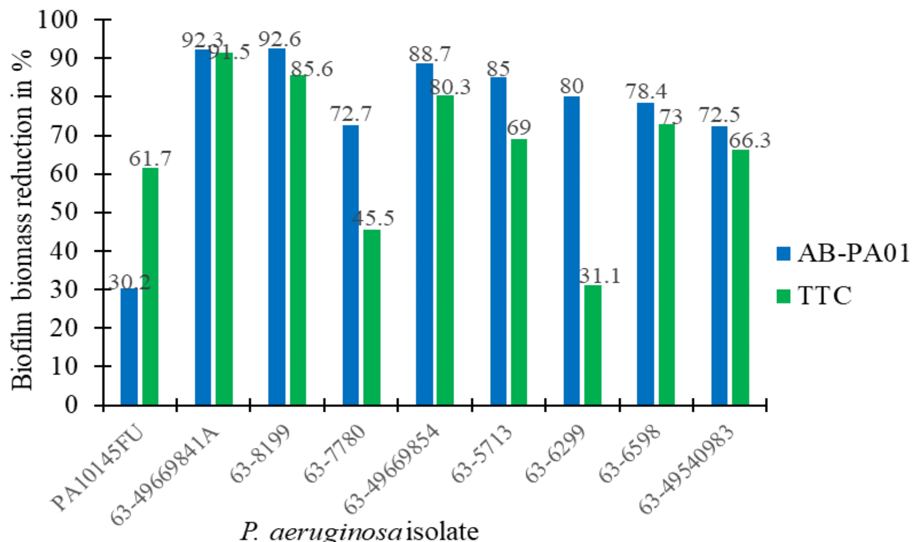

Figure 3. 7 Comparison of the **e**ffect of AB-PA01 and tetracycline (TTC) treatment on biofilm biomass reduction of *P. aeruginosa* isolates.

Treating biofilms with gentamicin did not produce statistically significant biofilm biomass reduction ($p > 0.05$; 1.4 vs 1.5) compared to PBS treatment, as shown in Figure 3.8.

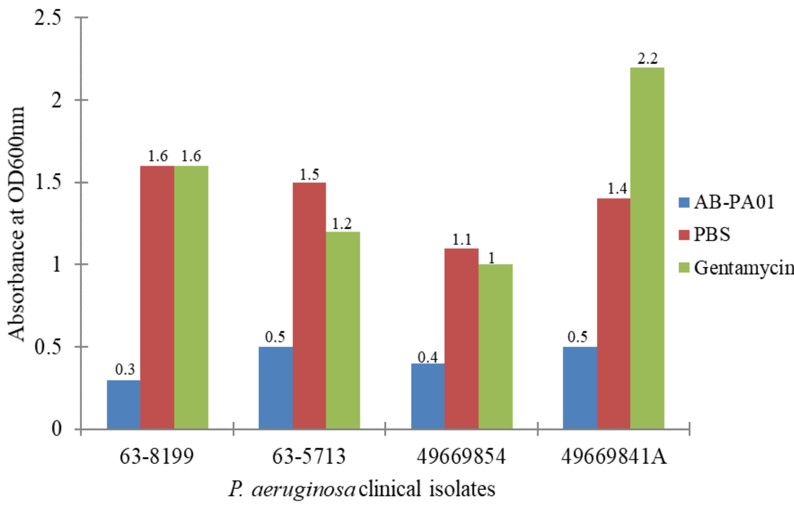

Figure 3. 8 Comparison of the effect of AB-PA01 and gentamicin treatment on *P. aeruginosa* isolates biofilm biomass reduction.

3.4 Discussion

P. aeruginosa is a leading causative agent of DFU infection among Gram-negative bacteria (Commons *et al.*, 2015; Lipsky *et al.*, 2010). In the warm and humid Asian and African countries, *P. aeruginosa* has been reported frequently to be a common causative microorganism of DFU infection (Wu *et al.*, 2017; Hatipoglu *et al.*, 2016). *P. aeruginosa* is also known for its persistent and aggressive infection (Lipsky *et al.*, 2010). Hence, a reliable, discriminatory, and quick identification method should be employed as *P. aeruginosa* isolates are inert for some biochemical tests and do not show unique phenotypic characteristics in the routine diagnostic tests (Kacaniova *et al.*, 2019). In this study, no Gram-positive bacterium was identified from the samples cultured on vancomycin-supplemented MacConkey agar, which agreed with previous reports (Myles *et al.*, 2016). In the literature, there is no consensus on the concentration of vancomycin to be included in the MacConkey medium to inhibit Gram-positive bacteria. However, from 4 to 64 mg/L vancomycin have been commonly used (Myles *et al.*, 2016; Brown and Walpole, 2003). In this study, the concentration of vancomycin added on the MacConkey agar was higher than the MICs found during VITEK® 2 tests to make the agar more selective.

MALDI-TOF MS analysis has been used extensively for microbial identification (Angeletti, 2017; Schumann and Maier, 2014). In this study, close to 90% of the suspected isolates were identified as *P. aeruginosa* species by MALDI-TOF MS. The identification score was ≥ 2.00 for all isolates that demonstrate high confidence of identification to the species level (Kacaniova *et al.*, 2019).

P. aeruginosa isolates show a significant degree of inherent resistance to many antibiotics such as β-lactams, tetracycline, aminoglycosides, fluoroquinolones, and sulfonamide mainly because of the low permeability of its outer membrane (Breidenstein *et al.*, 2011; Li *et al.*,

1994). Intrinsic antibiotic resistance mechanisms are the integral parts of the *P. aeruginosa* genome that require high MIC (Lister *et al.*, 2009). About 50% of the isolates tested in the present study were resistant to ticarcillin/clavulanic acid. Moreover, 10% - 15% of the isolates showed resistance against ciprofloxacin, cefepime, ceftazidime, and piperacillin/tazobactam. These antibiotic-resistant patterns of *P. aeruginosa* isolates are consistent with a previous report (Otto-Karg *et al.*, 2009).

This study was unable to confirm reports that showed significant proportions of *P. aeruginosa* clinical isolates are MDR (Sivanmaliappan and Sevanan, 2011; Falagas *et al.*, 2008) because only one MDR isolate was identified. In contrast to this study finding, a survey conducted on *P. aeruginosa* isolates collected from United Kingdom, Belgium, and Germany showed that 60% of the isolates were MDR (Mustafa *et al.*, 2016). Compared to *P. aeruginosa* isolates sampled from DFU patients that showed 50% – 100% resistance for all antibiotics they were exposed to in a study in India (Sivanmaliappan and Sevanan, 2011), the majority of the isolates tested in this study were susceptible for most of the antibiotics tested except ticarcillin/clavulanic acid.

Rate of antibiotic-resistant isolates was lower in this research, except for ticarcillin/clavulanic acid, than previous studies with cystic fibrosis from 3 European countries (Mustafa *et al.*, 2016), DFU from India (Sivanmaliappan and Sevanan, 2011), ventilator-associated pneumonia from France (Fihman *et al.*, 2015), nosocomial pneumonia from northern Europe (Riou *et al.*, 2010), and chronic rhinosinusitis from Australia (Fong *et al.*, 2017). In the present study, it was observed that amikacin, meropenem, tobramycin, norfloxacin, and gentamicin demonstrated a high level of antipseudomonal activity, and these antibiotics are better choices to treat DFU caused by *P. aeruginosa*. The rising prevalence of antibiotic-resistant pathogenic bacteria has prompted the search for alternatives such as lytic phages

(Golkar *et al.*, 2014). Effective phage therapy requires careful design through a multistep evaluation. Combining different phages in a formulation frequently results in a broader spectrum of antibacterial activity (Chan & Abedon, 2012).

In this study, both the *Myoviridae* and *Podoviridae* phages exhibited lytic activity over a broad range of clinical isolates, suggesting these phages are good candidates for therapy. Phage cocktail AB-PA01 possessed a superior host range compared with the most active single phage treatment in spot tests. As in the current study, the use of high phage titers (8 – 9 log_{10} (PFU/ml)) for host-range analysis is routine (Mendes *et al.*, 2014; Kutter, 2009). These individual phages and their cocktail used in the current study were effective against the higher bacterial inoculum (8 log_{10} (CFU/ml)) used to mimic heavily infected wounds (Mendes *et al.*, 2014). AB-PA01 and its components produces this effect at MOI 1 which is lower than the ‘multiplicity of 10 rule’, which is a guideline stating that if the goal is a significant reduction in bacterial density, it is better to use in the order of 10 phages to a bacterial host (Abedon, 2009; Kasman *et al.*, 2002). The broad host range and efficacy of the phage cocktail observed here is consistent with reports that the use of phage cocktails rather than individual phages improves phages activity by expanding the host range (Leskinen *et al.*, 2017; Mendes *et al.*, 2014; Chan *et al.*, 2013; Hall *et al.*, 2012).

During spot tests in the present study, it was found that the plaques of each phage and their combination were surrounded by opaque halo zones, which could be an indication of the presence of a phage-associated depolymerase as reported earlier (Cornelissen *et al.*, 2011). Halos are indications of enzymatic molecules diffusion and phages capability to disrupt extracellular polymerase substance of biofilms enzymatically (Gutierrez *et al.*, 2016; Sutherland *et al.*, 2004). Phage-associated depolymerase and related enzymes have been reported to enhance biofilm-reduction activity compared with non-depolymerase-inducing

phages (Hughes *et al.*, 1998). Although evaluation of phage lytic activity on a bacterial planktonic form is useful, biofilm studies should also be conducted because most infections involve biofilms (James *et al.*, 2008). Previous studies on *P. aeruginosa* suggest the potential of phages to reduce or eliminate biofilms (Alves *et al.*, 2016; Pires *et al.*, 2011) and prevent biofilm formation (Alves *et al.*, 2016; Fu *et al.*, 2010).

The effort to use gentamicin as a positive control in the present study was not successful as it produced no significant biofilm biomass reduction compared to the negative controls. Some gentamicin-treated *P. aeruginosa* isolates produced more biofilm biomass than control groups. This ineffectiveness of gentamicin in biofilm biomass reduction might be attributed to the fact that aminoglycosides often exhibit poor penetration through biofilms, resulting in reduced efficacy of gentamicin in biofilms (Ronan *et al.*, 2016; Stewart, 2002). The finding of this study was supported by a study that found the exposure of *P. aeruginosa* PAO1 biofilm to gentamicin alone did not result in any qualitative changes (Ronan *et al.*, 2016). The biofilm biomass reduction due to tetracycline treatment was similar to the AB-PA01 treatment effect.

In this study, phage cocktail AB-PA01 displayed a strong anti-biofilm activity on *P. aeruginosa* isolates biofilms *in vitro*. Over 60% of the isolates showed ≥ 50% biofilm biomass reduction compared to the negative control group, and of these, about 20% of the isolates showed ≥ 75% biofilm biomass reduction. This effect may be expected to increase with higher MOI because higher MOI produces a more significant biofilm reduction effect (Alves *et al.*, 2016). The lytic effect of AB-PA01 on bacterial host planktonic state translated well to a reduction in biofilms, consistent with earlier reports (Gutierrez *et al.*, 2016; Fu *et al.*, 2010). The biofilm reduction capability of AB-PA01 was not related to the antibiotic-resistance characteristics of the isolates. In general, the findings of the present study suggest

that phage cocktails could be feasible alternatives to antibiotic treatments in combating serious *P. aeruginosa* biofilm-related DFU infections.

In conclusion, *P. aeruginosa* isolates were identified at the species level by MALDI-TOF MS with high confidence of identification. The antibiotic susceptibility test conducted using VITEK® 2 demonstrated that *P. aeruginosa* isolates circulating in DFU patients of the current study settings had a good susceptibility pattern for most of the antibiotics tested except for ticarcillin/clavulanic acid. The isolates also displayed high susceptibility for each phage and the phage cocktail they were exposed to during spot test. The use of the phage cocktail broadened the host range and lysed most of the isolates resistant to some of its components. The strong lytic potential of AB-PA01 in the planktonic state is translated well to the biofilm biomass reduction since it exhibited significant anti-biofilm activity against *P. aeruginosa* isolates *in vitro.* Importantly, the lytic efficacy of AB-PA01 on host bacteria in planktonic state and anti-biofilm activity in biofilm phases was not related to the antibiotic-resistant pattern of *P. aeruginosa* isolates. Hence, AB-PA01 can be formulated as an alternative anti-infective therapeutic agent that may be useful in the treatment of biofilm-related DFU infection caused by *P. aeruginosa.*

Chapter 4

Efficacy of Phage Cocktails on *Staphylococcus aureus* and *Pseudomonas aeruginosa* in mixed-species culture

Chapter 4: Efficacy of Phage Cocktails on *Staphylococcus aureus* and *Pseudomonas aeruginosa* in mixed-species culture

Abstract

Antibiotic-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the commonly isolated bacteria from infected diabetic foot ulcers. Effective and easily available alternative therapeutic agents such as phages are needed. The efficacy of phages in mixed-species infections, especially in biofilms form, has been poorly examined. The *in vitro* lytic efficacy of phage cocktails AB-SA01, AB-PA01, and their combination was examined in *S. aureus* and *P. aeruginosa* mixed-species planktonic and biofilm cultures. Green fluorescent protein (GFP)-labelled *P. aeruginosa* PAO1, and mCherry-labelled *S. aureus* KUB7 laboratory strains and clinical isolates were used as target bacteria. During real-time monitoring of treatment effect using spectrophotometry, the density of mCherry *S. aureus* KUB7 and GFP *P. aeruginosa* PAO1 significantly decreased when treated by their respective phage cocktail, a mixture of phage cocktails, and gentamicin. The decrease in bacterial population measured by the reduction in fluorescence associated with the decrease in bacterial cell counts on selective agars. This mCherry- and GFP-based mixed-species microplate assay monitored through spectrophotometry combines reproducibility, rapidity, and ease of management. It is amenable to high-throughput screening for phage cocktail efficacy evaluation. Each phage cocktail, the combination of the two phage cocktails, and tetracycline produced significant biofilm biomass reduction in mixed-species biofilms. This study result shows that AB-SA01 and AB-PA01 lyse their hosts in the presence of non-susceptible bacteria, both in planktonic and biofilm states. The effect of each phage cocktail alone or in combination was more significant in planktonic cultures than in biofilms irrespective of host antibiotic resistance profile. These data support the use of phage therapy in polymicrobial infections.

4.1 Introduction

4.1.1 Antibiotic-resistant bacterial infection

Infections from antibiotic-resistant bacteria continue to threaten standard therapies and causing a global health crisis (Blair *et al.*, 2015). The proportion and number of MDR bacterial pathogens have increased in the last decade, and MDR bacterial infections are considered as emergent global diseases (Roca *et al.*, 2015). The direct impact of antibiotic-resistant bacterial infections includes a long duration of illness and increased mortality (Sowole *et al.*, 2018). It is estimated that in Europe, 25,000, and the United States, 23,000 people die each year as a result of MDR bacterial infections (Blair *et al.*, 2015). MDR *S. aureus* and *P. aeruginosa* are among the therapeutically challenging bacteria (Lebowitz *et al.*, 2017; Golkar *et al.*, 2014). DFU infections are usually treated with broad-spectrum antibiotics for prolonged durations (Uçkay *et al.*, 2015). The use of antibiotics for an extended time is one of the significant risk factors for antibiotic resistance (Harbarth *et al.*, 2015). In recent years, the selection of appropriate antibiotics for the treatment of DFU has been difficult due to the emergence of antibiotic-resistant pathogens (Lipsky *et al.*, 2016). This might require the identification of causative bacterial species and the development of specific treatments without the off-target effects.

4.1.2 Polymicrobial wound infections

The human body is a dynamic ecosystem naturally inhabited by a wide diversity of microorganisms that are collectively referred to as "microbiota" (Scalise *et al.*, 2015). The host immune system maintains the balance of these microorganisms under normal physiological conditions (Wong *et al.*, 2013). Skin, as part of the immune system, protects underlying tissues from harmful microorganisms. As an exposed organ, skin can be damaged

through injury, and healthy skin microflora may proliferate and cause inflammation (Scalise *et al.*, 2015). Damaged and exposed subcutaneous tissue may provide an environment conducive to microbial proliferation. The microflora associated with a small, clean, and well-perfused wound in an immunocompetent host may produce minimal effects, whereas the microflora associated with the presence of foreign material and devitalized tissue may facilitate microbial proliferation (Robson, 1997).

Wound infections, particularly chronic wound infections, are often polymicrobial. Multiple microorganisms in a single community can share genetic material, metabolic cooperation, quorum sensing systems, passive resistance, niche optimization, host immune modulation, and virulence induction that together facilitate microbial proliferation (Tay *et al.*, 2016; Shettigar *et al.*, 2016; Wolcott *et al.*, 2013). Bacteria such as *Enterococcus faecalis* modulate the polymicrobial niche by supplying nutritional elements to the co-infecting bacteria (Keogh *et al.*, 2016). Stable complex microbial communities are associated with extended wound healing period (Loesche *et al.*, 2017). The majority of polymicrobial chronic wound infections involve biofilms that further complicate treatment outcomes because bacteria in biofilm forms exhibit increased tolerance to antibiotic treatment (Pastar *et al.*, 2013; Dalton *et al.*, 2011; Bowler *et al.*, 2001).

Wound-infecting bacteria could originate from the environment, surrounding skin microflora, or endogenous sources (Bowler *et al.*, 2001). There is a controversy about the precise mechanism used by microorganisms to produce infection and the significance of their contribution towards non-healing wounds (Bertesteanu *et al.*, 2014). It appears that microbial load is an important factor in determining whether a wound heals (Robson, 1999; Raahave *et al.*, 1986). Polymicrobial infections are often linked with severe infection and poor treatment outcomes (Duplantier and van Hoek, 2013). Through acting synergistically, co-infecting

microbes can potentially increase their virulence (Tay *et al.*, 2016). Hence, polymicrobial infections may require new treatment strategies (Wolcott *et al.*, 2013).

4.1.3 Polymicrobial nature of DFU infections

Diabetes mellitus is a severe public health problem globally, and about 80% of diabetes-related lower extremity amputations are preceded by a foot ulcer (Hingorani *et al.*, 2016; Boulton, 2008). Most of the DFUs are infected, and the microbial burden is believed to contribute to poor healing (Prompers *et al.*, 2007). About one-third or more of DFU infections are found to be polymicrobial (Ramakant *et al.*, 2011). A recent study that investigated approximately 3,000 wounds found that only 7% of the wounds had single-species infections (Wolcott *et al.*, 2013). The polymicrobial bacterial communities in biofilms may contribute to delayed healing in DFUs (Mottola *et al.*, 2016). From polymicrobial DFUs infections, *S. aureus* and *P. aeruginosa* are commonly isolated microorganisms (Gjødsbøl *et al.*, 2006). DFU caused by *S. aureus* and *P. aeruginosa* is associated with increased wound severity and healthcare costs (Gjødsbøl *et al.*, 2006; Madsen *et al.*, 1996). A study by Mastropaolo and co-workers (2005), utilizing a diabetic mouse infection model, found that certain bacterial species significantly increased at the site of inoculation when co-infected with a second bacterium compared to a mono-species bacterial inoculation, indicating interspecies synergy (Mastropaolo *et al.*, 2005).

4.1.4 Role of *S. aureus* in DFU infection

It is estimated that 25% - 30% of adults are colonised with *S. aureus* (Schechter-Perkins *et al.*, 2011). In immunocompromised patients, or patients with potentially impaired wound healing, such as diabetes mellitus patients, *S. aureus* carriage can convert into a clinically evident infection (Cervantes-Garcia *et al.*, 2015; Lipsky *et al.*, 2011). The prevalence of *S.*

aureus in DFU patients varies in the range of 28–76% among Gram-positive bacteria (Eleftheriadou *et al.*, 2010).

S. aureus is found in about half of DFUs (Reveles *et al.*, 2016). Besides its abundance and antimicrobial resistance, *S. aureus* is virulent in DFUs (Richard *et al.*, 2011). Most of the *S. aureus* strains form a biofilm, which increases pathogenicity and contributes to treatment failure because it protects the pathogen from the effect of antibiotics and possibly impairs access to innate and adaptive immunity (Shettigar *et al.*, 2016; Vidlak and Kielian, 2016). *S. aureus* secretes virulence factors, including haemolysins, proteases, collagenases, and hyaluronidases, which help to necrotize host tissues and increase pathogenicity (Pfirman and Haile, 2018; Cosgrove *et al.*, 2003). Some *S. aureus* strains encode genes responsible for toxins production such as enterotoxins, toxic shock syndrome toxin-1 (TSST-1), exfoliative toxins A and B (*et*A and *et*B), and Panton-Valentine leucocidin, that are associated with high-grade infected diabetic ulcers and poor treatment outcomes (Viquez-Molina *et al.*, 2018; Sotto *et al.*, 2008).

4.1.5 Role of *P. aeruginosa* in DFU infection

Infection with MDR *P. aeruginosa* is increasing globally, primarily through nosocomial infections (Mesaros *et al.*, 2007). *P. aeruginosa* is the second most common pathogen found in DFUs after *S. aureus* (Sekhar *et al.*, 2018; Saltoglu *et al.*, 2018; Kateel *et al.*, 2018; Al Ayed *et al.*, 2018; Goldufsky *et al.*, 2015; Hatipoglu *et al.*, 2014). Due to its resistance to a wide variety of antibiotics via numerous antibiotic-resistance mechanisms, and the complexity of DFU, *P. aeruginosa* infection can be difficult to control through antibiotic treatment (Zhang *et al.*, 2014a; Mesaros *et al.*, 2007).

The presence of *P. aeruginosa* in wounds correlates with poor prognosis (Sivanmaliappan and Sevanan, 2011). Using its Type III Secretion System (T3SS), *P. aeruginosa* can cause severe and persistent infection in diabetic wounds (Goldufsky *et al.*, 2015). T3SS helps *P. aeruginosa* to release toxins, including ExoS, ExoT, ExoU, and ExoY, which can cause tissue damage, immune suppression, the spread of the bacteria, and inducing apoptosis of neutrophils (Goldufsky *et al.*, 2015; Zhang *et al.*, 2014a; Jia *et al.*, 2003). In studies with diabetic mouse wounds, infection with *P. aeruginosa* significantly increased the production of M1-phenotype macrophages which associates with the expression of pro-inflammatory and cytotoxic factors including tumour necrosis factor-a (TNF-a), interleukin-1 (IL-1), and interleukin-6 (IL-6) that may impair diabetic wound healing (Chen *et al.*, 2018).

4.1.6 *S. aureus* and *P. aeruginosa* co-infection in DFU

S. aureus and *P. aeruginosa* are commonly associated with chronic wound infections, as seen in DFUs (Trivedi *et al.*, 2014; Fazli *et al.*, 2009). The two pathogens can be found together in a non-random distribution in which *P. aeruginosa* occupies deeper regions of chronic wounds while *S. aureus* is found in the upper regions (Fazli *et al.*, 2009). *S. aureus* and *P. aeruginosa* form *in vivo* biofilms that contribute to antibiotic resistance of these bacterial species (DeLeon *et al.*, 2014). Difficulties in growing different bacterial species together *in vitro* make the study of co-infections challenging, and this is true in the case of *S. aureus* and *P. aeruginosa. P. aeruginosa* has been seen to kill *S. aureus* both in *in vitro* co-culture (Yang *et al.*, 2011; Machan *et al.*, 1991) and *in vivo* co-infection (Pernet *et al.*, 2014). This killing has been linked to various secretions of *P. aeruginosa* including LasA protease (Carnicero *et al.*, 1990), 4-hydroxy-2-heptylquinoline-N-oxide (HQNO) (Hoffman *et al.*, 2006), the pel and psl products (Qin *et al.*, 2009), and pyocyanin (Dietrich *et al.*, 2006).

Conversely, there are reports that *S. aureus* was not affected by *P. aeruginosa,* and the growth activity of *P. aeruginosa* was enhanced in the presence of *S. aureus* (Michelsen *et al.*, 2014). It is reported that *P. aeruginosa* was protected from autolysis, antibiotic effect, and host immunity by extracellular *S. aureus* proteins (Armbruster *et al.*, 2016). A commensal interaction between *S. aureus* and *P. aeruginosa* may occur in co-infection (Armbruster *et al.*, 2016; Michelsen *et al.*, 2014; DeLeon *et al.*, 2014). It has been shown that *P. aeruginosa* and *S. aureus* can stably exist in the *in vitro* wound environment and mutually benefit from the co-infection (DeLeon *et al.*, 2014).

4.1.7 *S. aureus* and *P. aeruginosa* co-culture

Studies suggest that *P. aeruginosa* forces the conversion of *S. aureus* to small colony variant (SCV) form with decreased metabolic activity in co-cultures (Filkins *et al.*, 2015; Mitchell *et al.*, 2010; Biswas *et al.*, 2009; Hoffman *et al.*, 2006). It is indicated that *S. aureus* survival in co-culture with *P. aeruginosa* is dependent on its ability to convert to the SCV phenotype (Hoffman *et al.*, 2006). The converted SCV *S. aureus* is often associated with increased tolerance to environmental stress (Moisan *et al.*, 2006). Furthermore, the presence of *P. aeruginosa* could activate staphyloxanthin producing mechanism of *S. aureus* white phenotypic variants, and it induces catalase that renders *S. aureus* more virulent and antibiotic-resistant (Antonic *et al.*, 2013). A recent study showed that timing and bacterial concentration of *S. aureus* and *P. aeruginosa* might affect the expression of quorum sensing genes and final bacterial ratio during mixed-species biofilm development (Woods *et al.*, 2018). Medium containing bovine serum albumin (BSA) is recommended to allow better growth of *S. aureus* in the presence of *P. aeruginosa* (Radlinski *et al.*, 2017; Kart *et al.*, 2014; Pernet *et al.*, 2014).

4.1.8 Effect of phages on mixed-species biofilms

Of all human infections, 65% - 80% are biofilm-related, and biofilm formation is often considered an underlying reason for antibiotic treatment failure (Coenye and Nelis, 2010). Bacteria residing in biofilms are more tolerant to physical and chemical disruptions than their planktonic counterparts (Steenackers *et al.*, 2016; Gilbert *et al.*, 2002). Studies have also shown that bacteria in biofilms are 100 -1000 times more resistant to antibiotics than in the planktonic phase (Burmolle *et al.*, 2006; Ceri *et al.*, 1999). Bacterial resistance to antibiotics in biofilms is mainly due to the complex nature of the EPS that gives mechanical stability to the biofilm and serves as an external digestive system that keeps extracellular enzymes near the bacteria (Flemming and Wingender, 2010). The EPS also protects bacteria since it reduces the access of solutes to the bacteria through the combination of ionic interaction and molecular sieving (Allison, 2003). The presence of metabolically inactive persister cells also contributes to the antibiotic-resistance of bacteria in biofilms (Harper *et al.*, 2014). Natural biofilms are usually polymicrobial sharing a common milieu and coexisting in niches by forming multispecies biofilms (Zhao *et al.*, 2013; Thein *et al.*, 2007).

Although many studies have examined the efficacy of phages on single-species biofilms, few have evaluated it on multispecies biofilms as discussed in a review (Geredew Kifelew *et al.*, 2019). There is a disparity among studies that evaluated the effect of phages on multispecies biofilms. Some reports showed that phages achieved a higher titre and depleted their bacterial host population effectively (Gonzalez *et al.*, 2017; Gutierrez *et al.*, 2015; Kay *et al.*, 2011; Sillankorva *et al.*, 2010). On the contrary, some reports indicated the inefficiency of phages within multispecies biofilms (Burmolle *et al.*, 2006; Tait *et al.*, 2002). This chapter aimed to examine the effect of *S. aureus* and *P. aeruginosa* phage cocktails, AB-SA01 and AB-PA01, on mixed-species planktonic and biofilm states.

4.2 Materials and methods

4.2.1 Bacteria

The bacteria used in this study were randomly selected four *S. aureus* and four *P. aeruginosa* isolates. From each species, one was a laboratory strain, and three were clinical isolates. The clinical isolates were collected from infected DFU patients at diabetic foot clinics in Adelaide, South Australia, during the study period by South Australia Pathology and generously provided to our laboratory on Muller Hinton agar culture. GFP-labelled *P. aeruginosa* PAO1 (PAO1) and mCherry-labelled *S. aureus* (*S. aureus* KUB7) (Burcham *et al.*, 2016) laboratory strains were generously donated by Dr. Nicky Thomas of University of South Australia, Australia, and A/Prof. Heather Jordan of Mississippi State University, USA, respectively.

The two fluorescent proteins are driven by constitutive promoters and do not need antibiotics to maintain expression. These were among the isolates that showed strong susceptibility in spot test and 73% - 88% biofilm biomass reduction on single-species biofilm experiments following treatment with the phage cocktail and its components, as discussed in chapter 2 and 3. A single colony of *P. aeruginosa* from a Muller Hinton agar plate was sub-cultured onto vancomycin-supplemented MacConkey agar (Thermo Fisher, South Australia), and *S. aureus* on MSA (Thermo Fisher, South Australia) as described (Mendes *et al.*, 2013; Kirisits *et al.*, 2005; Chapman, 1945). After 18 hours of incubation aerobically at 37 °C, the isolates were processed for identification and antibiotic susceptibility tests.

4.2.2 Phage cocktails

Phage cocktails AB-SA01 and AB-PA01 were donated by AmpliPhi Biosciences Corporation. AB-SA01 is a combination of J-Sa 36, Sa 83, and Sa 873 phages that belong to

the *Myoviridae*. AB-PA01 is a mixture of Pa 193 and Pa 204 from *Myoviridae,* and Pa 222 and Pa 223 from *Podoviridae*. None of these phage components encodes any known bacterial virulence or antibiotic resistance genes, and all phages were considered to be strictly lytic (Fong *et al.*, 2017; Lehman *et al.*, 2019). The phages are produced under cGMP standards and approved by the US Food and Drug Administration as an investigational new drug (Lehman *et al.*, 2019; Law *et al.*, 2019).

The phage titer determination was conducted on susceptible two laboratory strains and two clinical isolates for each phage cocktail using plaque assay following the established procedure (Mirzaei and Nilsson, 2015; Merabishvili *et al.*, 2009). The laboratory strains were *S. aureus* RN4220 and SA6538 for AB-SA01 and *P. aeruginosa* PAO1 and PA10145 for AB-PA01. The mean titers of AB-SA01 and AB-PA01 were 9.1 $\log_{10}$ (PFU/ml) and 10.3 $\log_{10}$ (PFU/ml), respectively.

4.2.3 Bacterial typing and antibiotic susceptibility testing

The bacterial isolates were identified using standard microbiology methods and MALDI-TOF MS biotyping (Bruker Daltonics Biotyper, Bruker Pty. Ltd., Victoria, Australia) as previously described (Lee *et al.*, 2015; Schumann and Maier, 2014; Lasch *et al.*, 2014; Harris *et al.*, 2010). The selective media MSA and vancomycin-supplemented MacConkey agar were used for *S. aureus*, and *P. aeruginosa* isolates, respectively. Antibiotic susceptibility pattern of the isolates was determined by VITEK® 2 (bioMérieux Australia Pty Ltd, New South Wales, Australia) as described earlier (Weber *et al.*, 2017; Gardiner *et al.*, 2013; Cartwright *et al.*, 2013; Ligozzi *et al.*, 2002).

4.2.4 *P. aeruginosa-S. aureus* co-culture test

Using the lab strains of *P. aeruginosa* (PAO1) and *S. aureus* (*S. aureus* KUB7), a pilot co-culture test was conducted both in planktonic and biofilm phases following established protocol with minor modifications (Gonzalez *et al.*, 2017; Kay *et al.*, 2011; Sillankorva *et al.*, 2010). For planktonic culture, each bacterial species was streaked on a nutrient agar plate and grown for 24 hours at 37 °C. From each bacterial species, 2 - 3 colonies were suspended in sterile physiological saline and adjusted to 1.0 McFarland turbidity standard (approximately 8.5 log_{10} cells). Each bacterial species' suspension was diluted in nutrient broth at 1:10 suspension-to-broth ratio. The two bacterial species cell suspensions were mixed in separate sterile test tubes at 1:1, 1:3, 1:5, and 1:7 *P. aeruginosa-to-S. aureus* ratio. Next, 50 µl of the mixture from each ratio was transferred to a culture tube containing 2.5 ml nutrient broth under sterile conditions.

The cultures in the tubes were grown in a shaker incubator at 37 °C and 160 rpm. After 18 hours incubation, 100 µl of the culture was mixed with nutrient soft agar pre-warmed to 42 °C and plated onto MSA and vancomycin-supplemented MacConkey agar plates. The plates were incubated at 37 °C for 24 - 48 hours, followed by manual counting of the CFU as described (Kumar and Ting, 2015). MacConkey agar was made more selective against *S. aureus* by adding vancomycin at 3 µl/ml of culture media based on VITEK® 2 antibiotic susceptibility test results. All *S. aureus* isolates used in this study were strongly susceptible to vancomycin.

Evaluation of the co-existence of *S. aureus* and *P. aeruginosa* in biofilms was conducted as described (Gutierrez *et al.*, 2015; Sillankorva *et al.*, 2010). Briefly: 200 µl of mixed broth culture from each ratio was transferred into wells of a 96-well flat-bottom microplate. The microplate was covered with aluminium foil and incubated at 37 °C in a static condition.

After 48 hours of incubation, the liquid culture was removed through gentle pipetting. The biofilm was then disrupted by aggressive scrapping of the bottom and wall of the wells using a sterile wire loop and collected using 200 µl nutrient broth. One hundred microliters of the suspension were mixed with nutrient soft agar pre-warmed to 42 ^{o}C and evenly plated onto MSA and vancomycin-supplemented MacConkey agar plates. The plates were incubated at 37 ^{o}C for 24 - 48 hours, followed by manual counting of the CFU. The experiment was conducted in triplicate on three different days.

4.2.5 Phage treatment of fluorescent mixed-species planktonic culture

Phage treatment on mixed-species bacterial planktonic cultures was carried out following an established protocol with minor modifications (De Jong *et al.*, 2017; Kumar and Ting, 2015). Two bacterial constructs, namely mCherry protein-labelled *S. aureus* KUB7 and green fluorescent protein-labelled *P. aeruginosa* PA01, were used. From the 18 hours of selective agar culture plates, 2 - 3 colonies were suspended in sterile PBS and adjusted to 1.0 McFarland equivalence turbidity standard, containing approximately 8.5 log_{10} (CFU/ml). Each bacterial species suspension was diluted at a ratio of 1:100 v/v with nutrient broth (Sigma-Aldrich, New South Wales, Australia) supplemented with 5% BSA) (Sigma-Aldrich, New South Wales, Australia) and incubated for 2 hours at 37 ^{o}C.

The two bacterial suspensions were then mixed at the ratio of 1:3 v/v of GFP PAO1 to mCherry *S. aureus* KUB7 in a sterile 10 ml test tube. This ratio of the two bacterial species was taken because it was from this ratio that equivalent bacterial density recovered during the pilot co-culture experiment. Two hundred microlitre of each mixture was transferred to a clear 96-well flat-bottom Greiner CELLSTAR® polystyrene tissue culture plate in triplicate (Sigma-Aldrich, New South Wales, Australia) and respective treatments were applied.

Mixed-species planktonic cell treatment were performed using five treatments: (i) a cocktail of *S. aureus* phage (AB-SA01), (ii) a cocktail of *P. aeruginosa* phage (AB-PA01), (iii) a mixture of *S. aureus* and *P. aeruginosa* phage cocktails (AB-SA01+AB-PA01), (iv) gentamicin, and (v) PBS treatment.

AB-SA01, AB-PAO1, or AB-SA01+AB-PAO1 phage cocktails were applied at the MOI of one to fluorescently labelled *S. aureus-P. aeruginosa* mixed-species culture. Gentamicin was used as a positive control at 16 µg/ml because its MIC for these isolates was ≤ 8 µg/ml during VITEK® 2 antimicrobial susceptibility test. An equal volume of PBS to phage solutions was applied to negative control groups. A plate cover was applied, and the plate was wrapped with aluminium foil from the top and sides. The plate was incubated in a CLARIOstar® Omega plate reader (BMG LABTECH Pty. Ltd., Victoria, Australia) for 24 hours at 37 °C with 100 rpm constant double-orbital shaking between measurements as described (Alves *et al.*, 2016).

The excitation and emission wavelengths were set at 570-15 and 620-20 nm for mCherry, and 470-15 and 515-20 nm for GFP detection, respectively. Fluorescence of mCherry and GFP were measured (in relative fluorescence unit, RFU) every 30 minutes in each well. Signals from triplicate wells were averaged and corrected for blank wells containing only nutrient broth, and the graph of fluorescence intensity was plotted. After 24 hours of incubation, bacterial colony counts were performed using serial dilution on selective agars for each species. The test was repeated three times using the same procedure on different days, and the mean was taken as final.

4.2.6 Phage treatment of clinical isolates mixed-species planktonic culture

A similar protocol with fluorescent mixed-species planktonic culture as above was followed for clinical isolates, except that incubation was in a standard incubator and no fluorescent intensity measurement. The effect of the treatment was evaluated using bacterial density reduction by colony count method.

4.2.7 Phage treatment of *in vitro* mixed-species biofilm

The mixed-species biofilm development and treatment were conducted as described (Gonzalez *et al.*, 2017; Mendes *et al.*, 2014) with few modifications. Briefly, 2 - 3 colonies of 18 hours culture of each isolate were independently suspended in sterile physiological saline and adjusted to 1.0 McFarland turbidity standard. These suspensions were pooled at a 1:3 v/v ratio of *P. aeruginosa*-to-*S. aureus*, and 100 µl of the pooled suspension was transferred to 10 ml 5% BSA nutrient broth. The final suspension was supplemented with 1% sterile glucose to facilitate biofilm development. Two hundred microlitre of the suspension was transferred into a tissue culture plate in triplicate and incubated for 48 hours at 37 ^{o}C with 70 rpm agitation.

After 48 hours of incubation, the liquid culture was removed, and plates were washed gently twice using sterile deionized water. Then, 225 µl of AB-SA01, AB-PAO1, or AB-SA01+AB-PAO1 suspension in the nutrient broth was applied to the respective treatment group biofilms. The treatment categories of mixed-species biofilms were: (i) *S. aureus* phage cocktail AB-SA01, (ii) *P. aeruginosa* phage cocktail AB-PA01, (iii) a mixture of the two phage cocktails, AB-SA01+AB-PA01, (iv) tetracycline (positive control), and (v) PBS (negative control).

The effect of each phage cocktail and a combination of the two phage cocktails was compared to tetracycline- and PBS-treated groups. Tetracycline was used as a positive control in mixed-species biofilm treatment as it was strongly effective ($p < 0.001$), compared

with PBS treatment, in biofilm biomass reduction on single-species biofilm treatment of both *S. aureus* and *P. aeruginosa* isolates as discussed in chapters 2 and 3 of this thesis. Gentamicin did not produce significant biofilm biomass reduction ($p > 0.05$). Tetracycline was not used as a positive control in mixed-species planktonic culture treatment experiments to avoid exaggerated fluorescence detection because of its color and fluorescence nature (Ni *et al.*, 2010).

MOI of each phage cocktails or their mixture was one based on each bacterial species density recovered during the co-culture pilot experiment. An equivalent volume of tetracycline and PBS to phage solution in nutrient broth were also applied as controls. The concentration of tetracycline was 128 µg/ml because *P. aeruginosa* isolates are susceptible to a higher concentration of tetracycline (Zheng *et al.*, 2017; Pai *et al.*, 2001). The MIC of tetracycline for *S. aureus* was ≤ 8 µg/ml during VITEK® 2 antimicrobial susceptibility test. Treated biofilms were incubated for 12 hours at 37 ^{o}C at static conditions. The biofilm was washed twice using 250 µl sterile PBS through careful pipetting. The biofilm-associated cells attached to the well surface were collected with 225 µl nutrient broth through pipetting after scraping the wall and bottom of the wells with a loop as described (Gutierrez *et al.*, 2015; Sillankorva *et al.*, 2010). After homogenization with a vortex mixer, the cell suspension was serially diluted, 10^{-1} - 10^{-8}, in filter-sterilized 10 mM ferrous ammonium sulphate (FAS) supplemented nutrient broth to inactivate free phages (McNerney *et al.*, 1998). The diluted suspension was incubated at room temperature for 15 minutes.

4.2.8 Colony count

Bacterial quantification was performed using the 10-fold serial dilution method (O'Toole, 2016; Mendes *et al.*, 2013). One hundred microlitres of the bacterial suspension from each serial dilution were mixed with nutrient soft agar warmed at 42 ^{o}C and dispensed over 37 ^{o}C

pre-warmed MSA and vancomycin-supplemented MacConkey agar in triplicate and incubated at 37 ^{o}C for 24 hours. Plates with approximately 30-300 colonies were taken from one of the dilutions, and colony count was carried out as described (O'Toole, 2016). The bacterial cell was calculated using the formula B = N/d, where B = number of bacteria, N = average number of colonies counted on the three plates, and d = dilution factor as described earlier (Mendes *et al.*, 2013). The results are expressed as logarithm-transformed values (log_{10} (CFU/ml)).

4.2.9 Data management and statistical analysis

Data were double entered, encoded, and stored using Microsoft Excel Spreadsheet. STATA version 16 software was used for statistical analysis. Data are reported in terms of mean and are expressed as logarithm-transformed values log_{10} (CFU /ml) over time. A comparison of experimental groups was performed using a one-way analysis of variance (two-tailed) or paired ‘t-*test*’. A $p < 0.05$ value was considered statistically significant.

4.3 Results

4.3.1 Colony count result of co-culture tests

The capability of *S. aureus* KUB7 and *P. aeruginosa* PA01 to grow in planktonic culture and establish biofilm in the presence of each other was examined. The outcome of the co-culture study was evaluated by taking samples from mixed-species cultures and plating onto selective agars and performing colony counts. The results confirmed that *S. aureus* KUB7 and PA01 co-exist in planktonic and biofilm cultures. The mean bacterial population in planktonic and biofilm phases at 1:3 volume-to-volume PA01-to-*S. aureus* KUB7 ratio was 7 $\log_{10}$ (CFU/ml) for each species. At 1:1 ratio, the mean CFU for *S. aureus* KUB7 was 5 $\log_{10}$ (CFU/ml) while PA01 was 7 $\log_{10}$ (CFU/ml). When *S. aureus* KUB7 concentration increase beyond 1:3 PAO1-to-*S. aureus* KUB7 ratio, CFU of *S. aureus* KUB7 increased, but the CFU of PAO1 decreased. Moreover, *S. aureus* KUB7 and PAO1 mixed planktonic culture monitored by CLARIOstar spectrophotometer for 24 hours showed similar fluorescence detection and CFU results during colony count. Hence, a 1:3 *P. aeruginosa*-to-*S. aureus* ratio was selected for the subsequent mixed-species culture treatment experiments.

4.3.2 Phage cocktail treatment on fluorescent mixed-species planktonic culture

The effects of the treatments were evaluated by measuring fluorescence, and bacterial cell count of each species on selective agar medium. The fluorescence of each species alone or together was confirmed under a confocal microscope, as shown in Figures 4.1**A** – 4.1**C**. No growth differences were observed between the mCherry *S. aureus* KUB7, GFP PAO1, and clinical isolates using the OD reading and CFU count in biofilm and CFU count in plate cultures. The magnitude of the fluorescence detected (in terms of relative fluorescence unit

(RFU)) at each time point of measurement for 24 hours demonstrated the efficacy of the treatments.

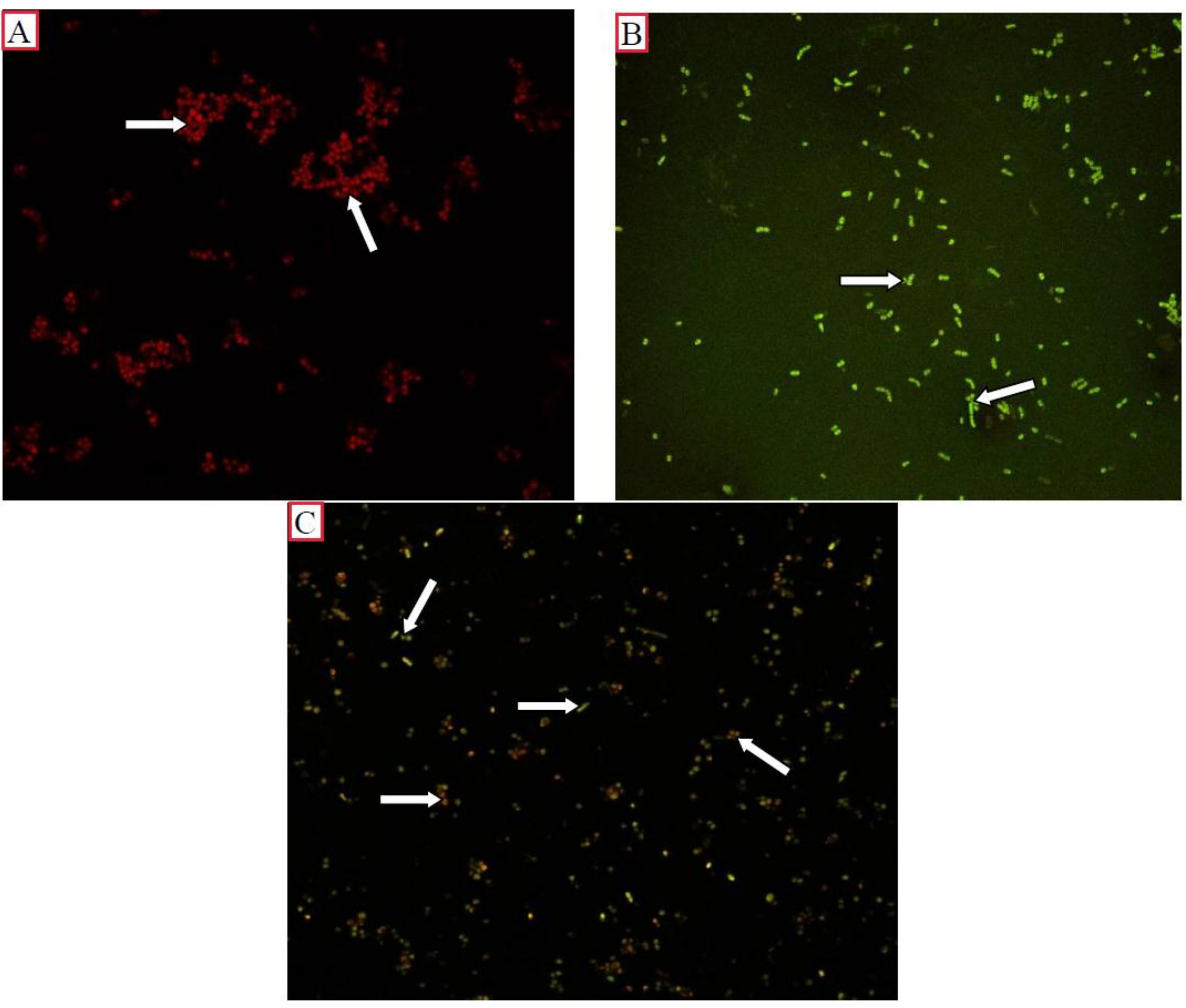

Figure 4. 1 Fluorescence strain under confocal microscopy: **A -** mCherry labelled *S. aureus* KUB7 (red data points) alone, **B** - GFP labelled *P. aeruginosa* PAO1 (green data points) alone, and **C** - mCherry labelled *S. aureus* KUB7 mixed with GFP labelled *P. aeruginosa* PAO1.

In single-species cultures treated with PBS, there was a marked increase in fluorescence of mCherry SA KUB7 and GFP PAO1, as shown in Figures 4.2**A** and 4.2**B**, indicating bacterial growth. In the mixed-species cultures without phages, the magnitude of fluorescence slowly increased with time (Figure 4.2**C**). However, the maximum red and green fluorescence obtained was much lower than the fluorescence detected during single-species PBS-treated cultures (Figures 4.2**A** and 4.2**B**), suggesting that the mixed-species exhibited co-inhibitory

effects. In mixed-species cultures treated with a single phage cocktail, the fluorescence of the target host was almost eliminated, while the non-target host was unaffected, as shown in Figures 4.2**D** and 4.2**E**, with fluorescence similar to PBS-treated single-species control.

When both phage cocktails were added to the mixed-species cultures, there was low fluorescence of both bacterial species, as shown in Figure 4.2**F**, similar to the inhibitory effect of gentamicin (Figure 4.2**G**), indicating that phage efficacy is not affected by the presence of non-host bacteria or other phages. The highest magnitude of red fluorescence in mCherry SA KUB7 was detected from the untreated single-species culture (Figure 4.2**A**), but in the case of GFP PAO1, the highest green fluorescence was detected from mixed-species culture treated with *S. aureus* phage cocktail, AB-SA01 (Figure 4.2**D**). The lowest magnitude of fluorescence from the target host in the mixed-species culture was observed when treated with each phage cocktail, as shown in Figures 4.2**D** and 4.2**E**. As expected, AB-SA01 and AB-PA01 exhibited no lytic effect on non-susceptible hosts (Figures 4.2**D** and 4.2**E**). While the fluorescence detected from a non-susceptible host showed an increase through time, the fluorescence obtained from the susceptible host remained low.

The decreases in fluorescence from each phage cocktail-, combinations of the two phage cocktails-, and gentamicin-treated groups were significantly lower compared to the PBS-treated group (Figures 4.2**A** - 4.2**G**). The corresponding colony count results for each treatment group after 24 hours are shown in Table 1 and confirm the results obtained with the fluorescence detection methodology.

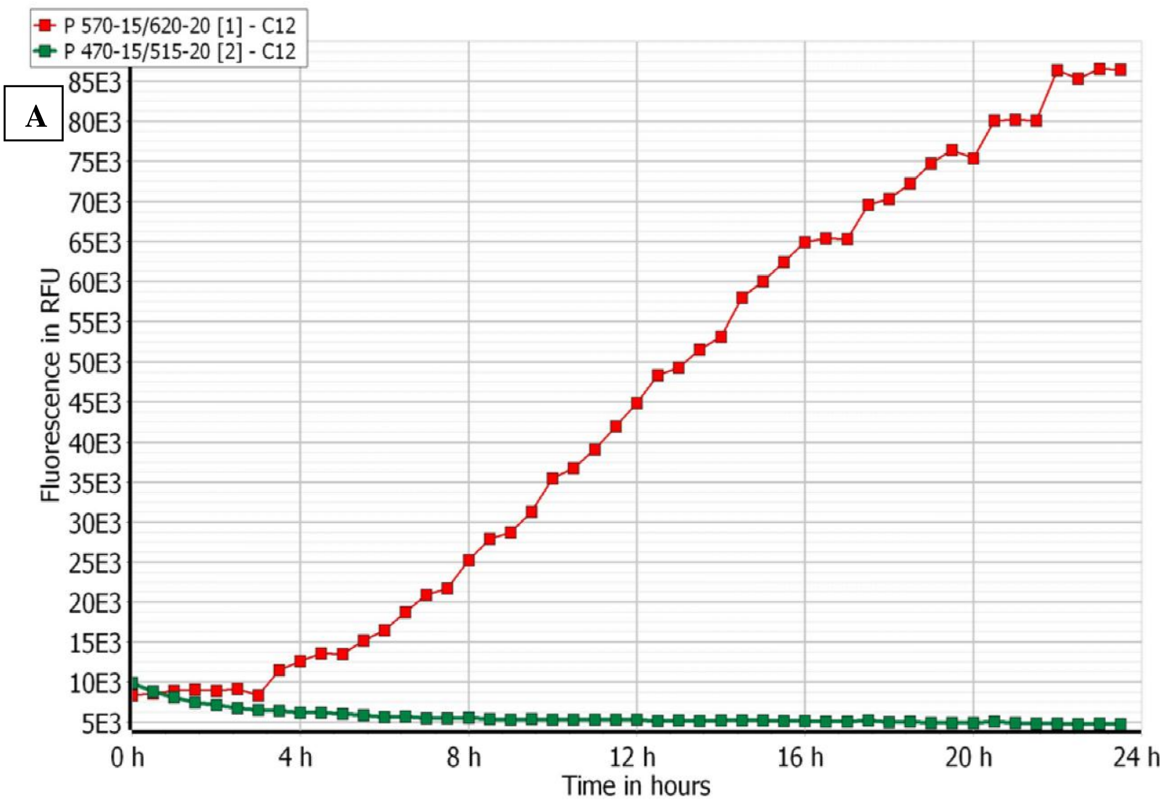

Figure 4. 2 PBS-treated mCherry *S. aureus* KUB7 single-species culture. The green graph is due to background detection since the machine was set for mCherry and GFP detection.

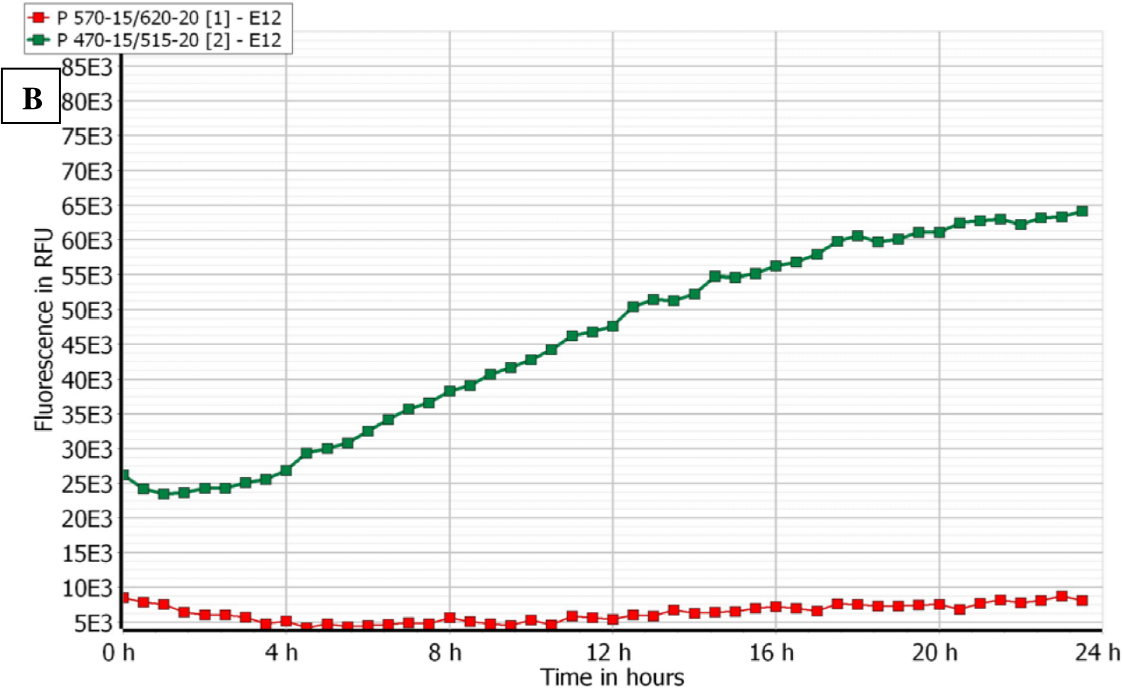

Figure 4. 2B PBS-treated GFP PAO1 single-species culture. The red graph is due to background detection since the machine was set for mCherry and GFP detection.

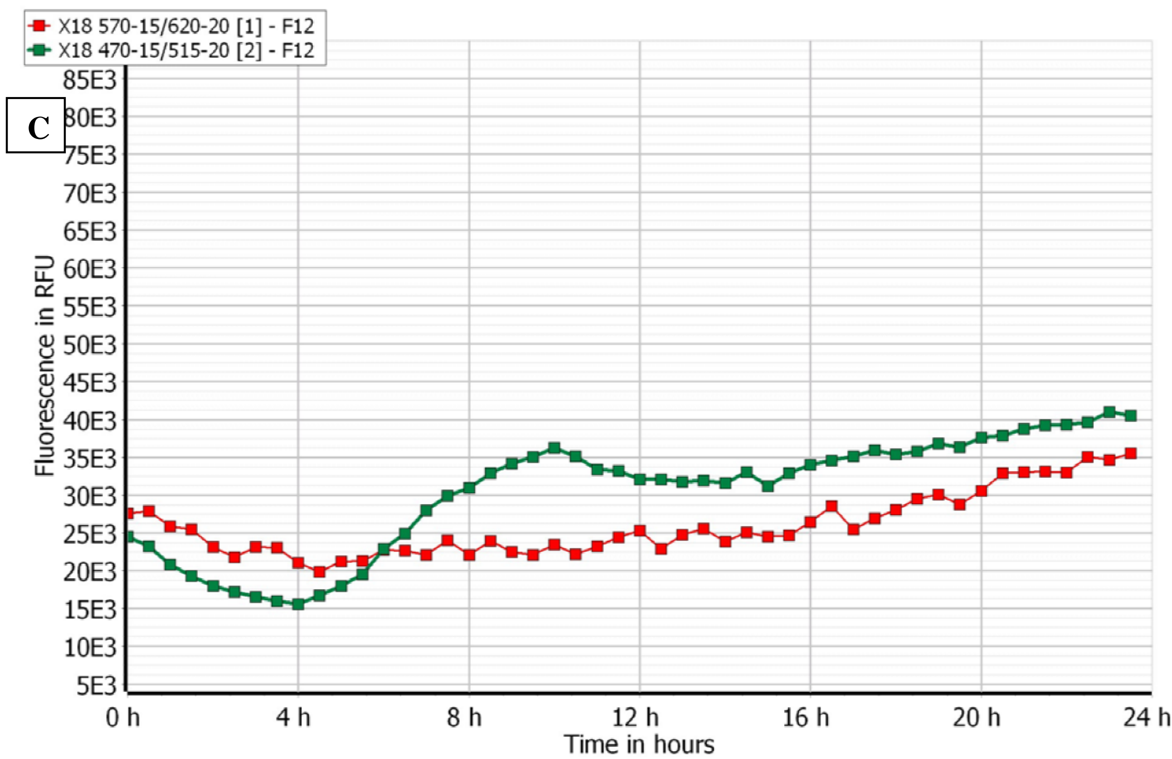

Figure 4. 2C PBS-treated mCherry *S. aureus* KUB7 and GFP PAO1 mixed-species culture.

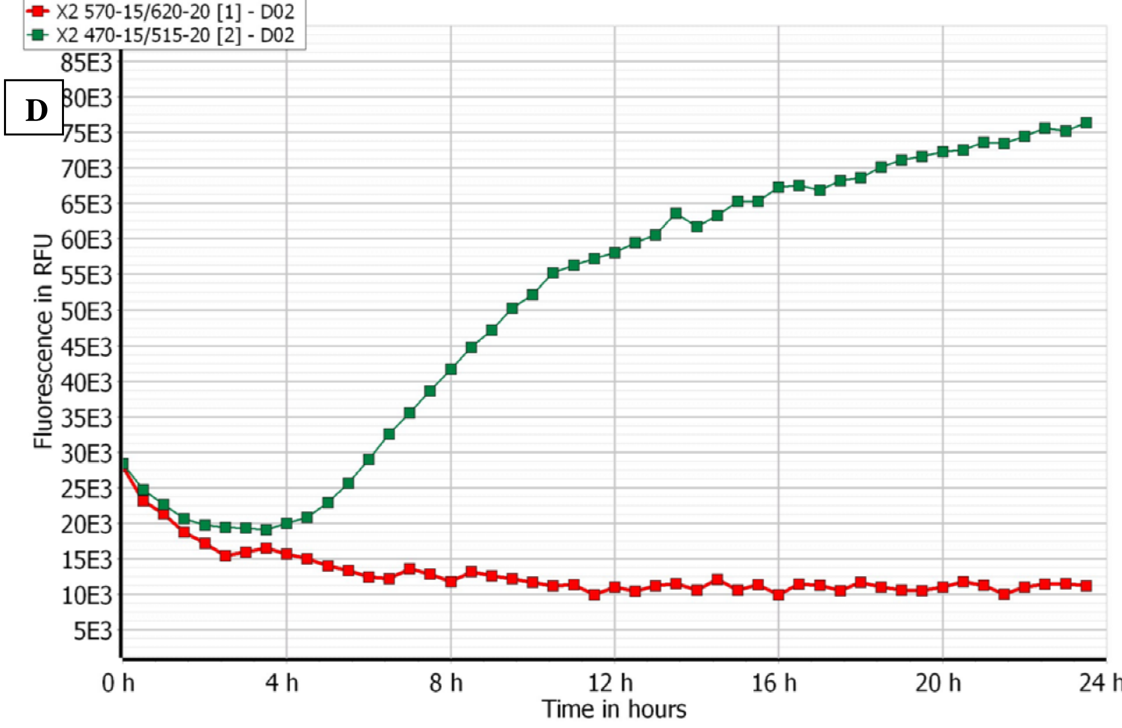

Figure 4. 2D AB-SA01-treated mCherry *S. aureus* KUB7 and GFP PAO1 mixed-species culture.

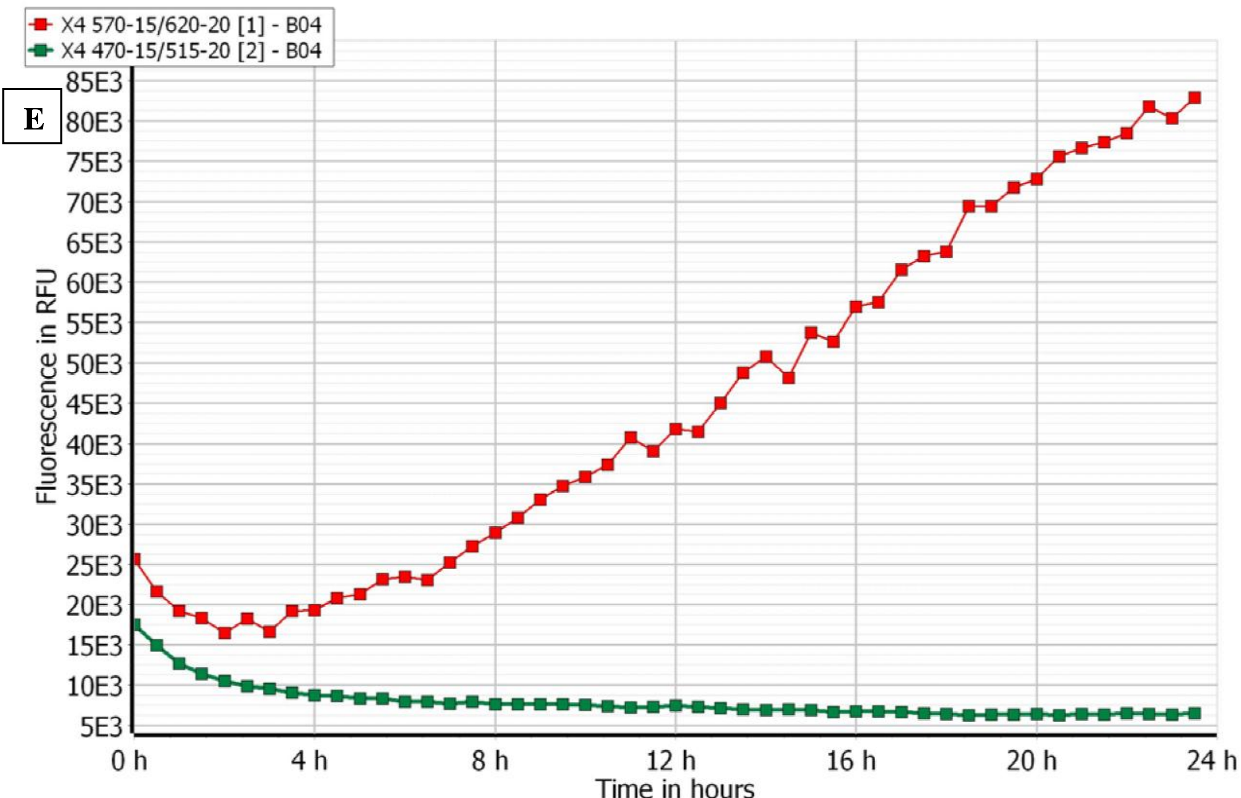

Figure 4. 2E AB-PA01-treated mCherry *S. aureus* KUB7 and GFP PAO1 mixed-species culture.

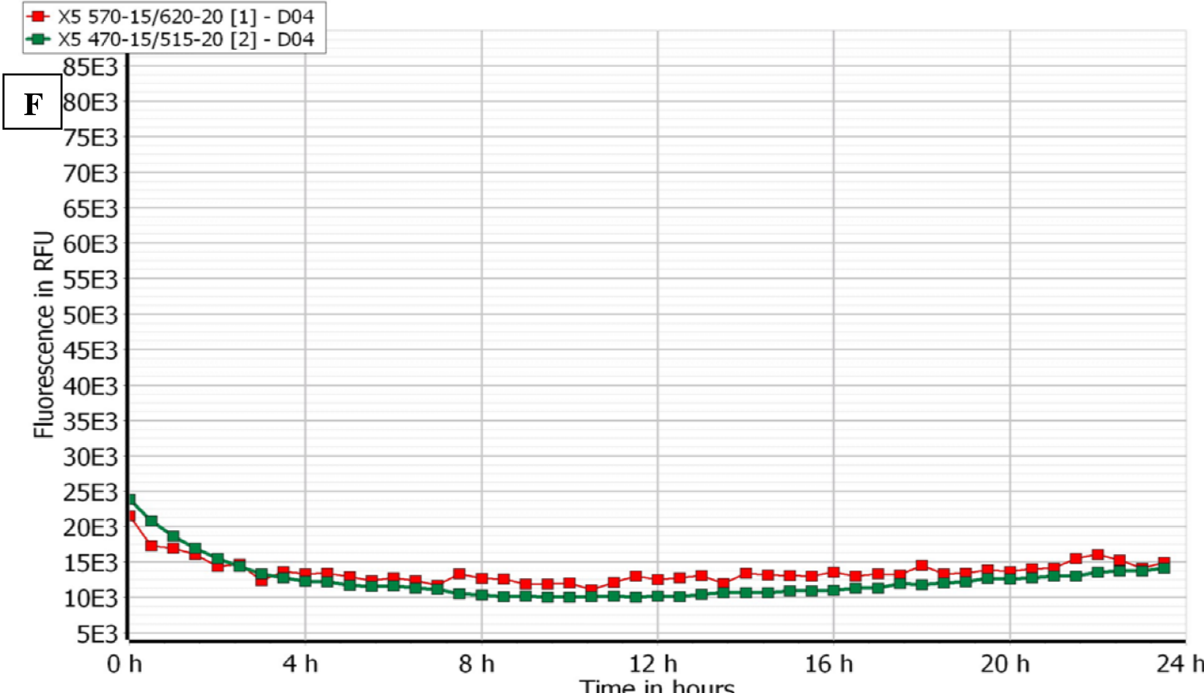

Figure 4. 2F AB-PA01+AB-SA01-treated mCherry *S. aureus* KUB7 and GFP PAO1 mixed-species culture.

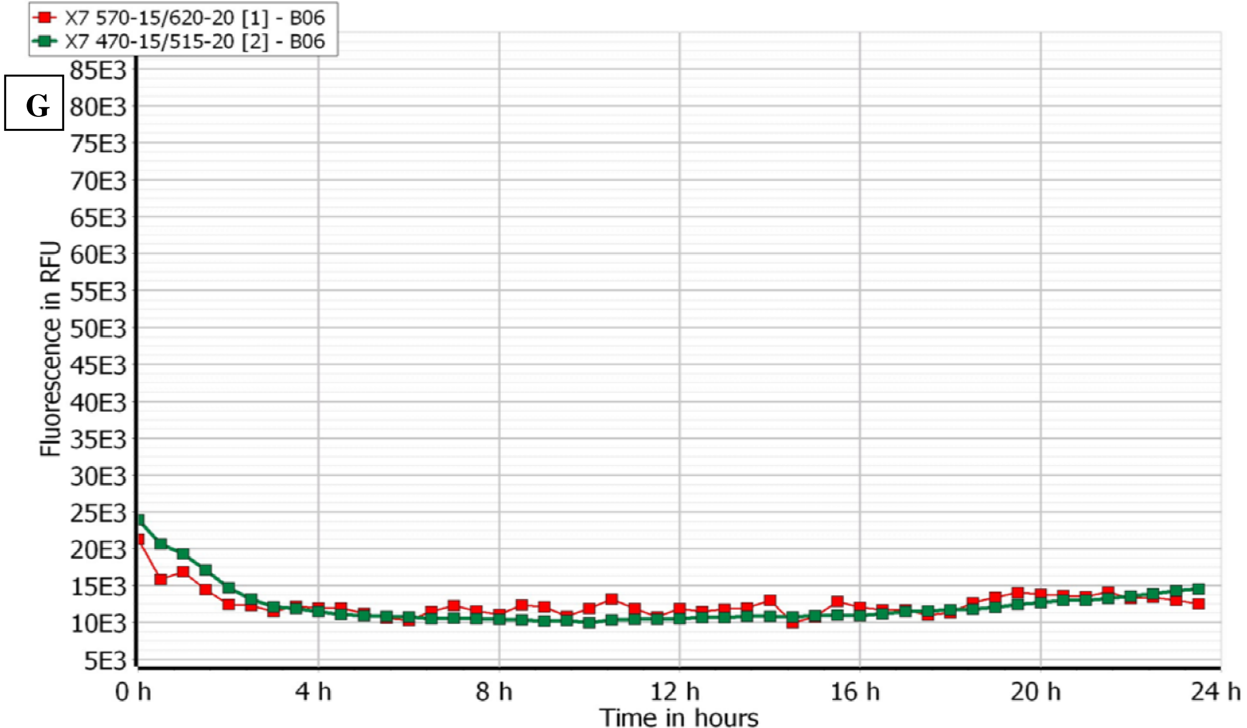

Figure 4. 2G Gentamicin-treated mCherry *S. aureus* KUB7 and GFP PAO1 mixed-species culture.

Figure 2A - 2G: Effect of phage cocktails on mCherry S. aureus KUB7 (red data points) and GFP P. aeruginosa PAO1 (green data points) single- and mixed-species planktonic cultures. RFU represents relative fluorescence unit.

4.3.3 Efficacy of phage cocktails on laboratory and clinical isolates mixed-species planktonic culture

The population of each bacterial species in mixed-species planktonic cultures at the end of 24 hours of treatment was assessed. Compared to PBS-treated samples, AB-SA01- and AB-PA01-treated samples yielded a significantly lower bacterial population ($p < 0.001$), which is 3.3 $\log_{10}$ (CFU/ml) and 5.1 $\log_{10}$ (CFU/ml) reduction on their hosts, respectively, as shown in Table 4.1. When the same samples were treated by the combination of the two phage cocktails, AB-SA01+AB-PA01, the mean cell count of *S. aureus* and *P. aeruginosa* reduced by 4.7 $\log_{10}$ (CFU/ml) and 3.8 $\log_{10}$ (CFU/ml), respectively. The cell counts of one bacterial species showed an increase when the culture was treated with only a phage cocktail of the

other species in the mixed-species culture. All planktonic cultures treated with gentamicin yielded no visible bacterial cells.

Table 4. 1 Bacteria cell count (log_{10} (CFU/ml)) of *S. aureus* and *P. aeruginosa* in mixed-species planktonic culture after 24 hours phage cocktails, gentamicin, or PBS treatment.

Isolates Combination	Evaluated isolate	Bacterial cell counts after treatment				
		PBS	AB-SA01	AB-PA01	AB-SA01+ AB-PA01	Gentamicin
S. aureus KUB7^S	*S. aureus* KUB7^S	5.5	3.6	8.7	3.0	0
and PA01 GFP^P	PA01 GFP^P	7.9	8.9	0	3.7	0
63-6538^S and 63-	63-6538^S	5.6	1.5	7.9	0	0
6598^P	63-6598^P	5.3	6.0	0	0	0
63-2498^S and 63-	63-2498^S	6.2	0	6.6	0	0
5497^P	63-5497^P	7.5	8.0	3.5	4.0	0
63-5656^S and 63-	63-5656^S	4.8	3.6	6.1	0	0
6036^P	63-6036^P	6.6	8.1	3.3	4.2	0
Summarized treatment effect						
	S. aureus mean	5.5	2.2	7.3	0.8	0
	S. aureus reduction	---	3.3	+ 1.8	4.7	5.5
	P. aeruginosa mean	6.8	7.8	1.7	3.0	0
	P. aeruginosa reduction	---	+ 1.0	5.1	3.8	6.8

Key: ^{S} *S. aureus* isolates, ^{P} *P. aeruginosa* isolates, + indicates an increase in bacterial count compared to PBS treatment.

When the two phage cocktails were applied together, the mean *S. aureus* CFU reduction was 1.4 log_{10} (CFU/ml) more than the reduction caused by AB-SA01 alone. On the contrary, AB-PA01 became less effective when applied in combination with AB-SA01 than alone. During mixed phage cocktails treatment, AB-PA01 produced 1.3 log_{10} (CFU/ml) less effect compared to AB-PA01 only treatment. The increases of *S. aureus* and the decrease of *P. aeruginosa* during AB-SA01+AB-PA01 treatment was consistent across each bacterial species isolate tested. Each bacterial species was seen to grow better when the other species was removed from mixed-species culture by the phage of the other bacteria. When all the

eight bacterial isolates (four *S. aureus* and four *P. aeruginosa*) culture treated with gentamicin, no bacterial cell was detected from any of the isolates.

4.3.4 Efficacy of phage cocktails on mixed-species biofilms

The findings of this study demonstrate that phage cocktails AB-SA01, AB-PA01, and a mixture of AB-SA01 and AB-PA01 successfully lysed their hosts in the presence of biofilms of non-susceptible species. These phage cocktails applied to *S. aureus* and *P. aeruginosa* mixed-species biofilms caused a statistically significant reduction in the host cell population compared to the PBS-treated group ($p < 0.05$; Table 4.2). However**,** the reduction of the cell population in *S. aureus* and *P. aeruginosa* was less by half than the decrease observed in planktonic culture treatment. Most of the tetracycline-treated cultures produced no viable bacterial cells of the clinical isolates and low bacterial cell density of the laboratory strains.

Table 4. 2 Bacteria count (log_{10} (CFU/ml)) of *S. aureus* and *P. aeruginosa* in mixed-species biofilms after 24 hours phage cocktails, tetracycline, and PBS treatment.

Isolates Combination	Evaluated isolate	Bacterial cell counts after treatment				
		PBS	AB-SA01	AB-PA01	AB-SA01+ AB-PA01	Tetracycline
S. aureus KUB7^S and PA 01 GFP^P	*S. aureus* KUB7^S	6.2	4.5	7.4	5.5	3.8
	PA01 GFP^P	6.4	6.3	3.8	4.0	3.9
63-6538^S and 63-6598^P	63-6538^S	5.2	4.4	4.7	3.6	3.0
	63-6598^P	5.5	5.6	3.6	3.7	0
63-2498^S and 63-5497^P	63-2498^S	6.3	4.4	5.2	5.5	0
	63-5497^P	7.1	6.2	5.5	5.2	0
63-5656^S and 63-6036^P	63-5656^S	7	4.9	5.9	5.3	0
	63-6036^P	8.5	8.9	4.7	6.3	0
Summarized treatment effect						
	S. aureus mean	6.2	4.6	5.8	5.0	1.7
	S. aureus reduction	----	1.6	0.4	1.2	4.5
	P. aeruginosa mean	6.9	6.8	4.4	4.8	1.0
	P. aeruginosa reduction	----	0.1	2.5	2.1	5.9

Key: ^{S} *S. aureus* isolates, ^{P} *P. aeruginosa* isolates.

Compared to PBS treatment, the application of AB-SA01 or AB-PA01 alone did not produce a statistically significant effect on the cell count of the non-host bacterial species population ($p > 0.05$; 6.9 vs 6.8 for AB-SA01 and 6.2 vs 5.8 for AB-PA01, Table 4.2). The mean bacterial cell population of each species remained unaffected when treated with the other species' phage cocktail alone. Treatment of mixed-species biofilms using the mixture of the two phage cocktails, AB-SA01+AB-PA01, produced similar cell reduction on both *S. aureus* and *P. aeruginosa* isolates as each phage cocktail treatment. The effect of tetracycline treatment caused a significant reduction in the population of both bacterial species.

4.4 Discussion

The two antibiotic-resistant bacterial pathogens commonly isolated from diabetic wound infections are *S. aureus* and *P. aeruginosa* (Ibberson *et al.*, 2017; Trivedi *et al.*, 2014; Fazli *et al.*, 2009). The rationale to examine the effect of phage cocktails treatment on mixed-species planktonic and biofilm cultures in the present study was that mixed-species biofilms cause many wound infections (Wolcott *et al.*, 2013; Ramakant *et al.*, 2011), and the efficacy of phages in such settings has been poorly examined. In polymicrobial infections, interspecies interactions range from antagonism to cooperation that can significantly impact the pathogenicity of microbes and clinical outcomes of the infection (Antonic *et al.*, 2013; Hoffman *et al.*, 2006).

In *P. aeruginosa* and *S. aureus* co-infections, HQNO produced by *P. aeruginosa* impairs *S. aureus* growth (Hoffman *et al.*, 2006) and facilitates the use of important *S. aureus* metabolites by *P. aeruginosa* (Mashburn *et al.*, 2005). HQNO is a molecule that inhibits Gram-positive bacteria respiration (Kharel *et al.*, 2004). Conversely, it was reported that HQNO selects for aminoglycoside-resistant *S. aureus* SCVs (Hoffman *et al.*, 2006) and protects *S. aureus* from stressful conditions such as oxidative stress in cystic fibrosis co-infection and co-culture with HQNO-producing *P. aeruginosa* (Antonic *et al.*, 2013). Thus, in *P. aeruginosa*-*S. aureus* co-infections, SCVs may be missed by clinical laboratories because of their slow growth and atypical phenotype (Hoffman *et al.*, 2006) and are capable of intracellular growth to cause chronic infections (Proctor *et al.*, 2006).

A co-infecting *P. aeruginosa* can activate some *S. aureus* virulence factors such as staphyloxanthin and catalase via inter-species communication (Antonic *et al.*, 2013). In this study, a mixed-species culture of mCherry-labelled *S. aureus* and GFP-labelled *P. aeruginosa* laboratory strains was used to examine the phage treatment effect by measuring the

magnitude of fluorescence of the two species within fixed time intervals for 24 hours by fluorescence spectrophotometry, with the results discussed below.

The death of a bacterial cell has been defined as the inability of a cell to grow to a detectable size or number on culture media (Berney *et al.*, 2007). Monitoring of the bacterial growth pattern using fluorescence detection at regular intervals, biofilm measurement using absorbance at OD_{600} nm, and CFU count results in the current study suggest that expression of fluorescent proteins does not negatively affect the growth fitness and viability of the fluorescently labelled strains (Burcham *et al.*, 2016; Barbier and Damron, 2016; Pereira *et al.*, 2010).

While light, fluorescence, confocal, and electron microscopy have been used to visualize cells in planktonic and biofilm cultures, microplate reader spectrophotometry allows the ready detection of fluorescently marked cells (Johnson *et al.*, 2015). The use of fluorescent proteins is common in microscopy and flow cytometry analysis (Pereira *et al.*, 2010; Briandet *et al.*, 2008; Hoerr *et al.*, 2007; Armstrong and He, 2001; Nebe-von-Caron *et al.*, 2000). However, the use of fluorescing proteins, particularly multiple fluorescent proteins simultaneously, in plate reader spectrophotometry, is limited.

Live cell examination techniques are essential tools to better understand microbial organization and function *in vitro* and *in vivo*. Multiple fluorescent proteins can be simultaneously applied to examine different microbial population interactions and to evaluate antimicrobial treatment effects in real-time (Lagendijk *et al.*, 2010; Chudakov *et al.*, 2005). The use of red fluorescent protein (mCherry) in combination with a green fluorescent protein (GFP) is suitable as the excitation and emission spectra of these proteins are well separated (Lagendijk *et al.*, 2010; Malone *et al.*, 2009; Matz *et al.*, 1999). The advantages of using mCherry and GFP as markers include ease of detection, no exogenous substrate is required

that may perturb biological samples, no need for cell processing to enable visualization, and the appropriateness for real-time monitoring of cells growth in terms of fluorescence intensity in culture (Vickerman *et al.*, 2015; Pereira *et al.*, 2010).

In this study, mCherry *S. aureus* KUB7 and GFP PAO1 were used to distinguish them in mixed-species culture. Loss of mCherry or GFP fluorescence from the respective bacterial population was considered an indicator of bacterial cell death due to the treatment effect. Previous studies demonstrated that the decrease in fluorescence is because of the cell death, not due to the degradation of the proteins, showing this fluorescence detection technique to be an early and sensitive marker of cell death (Steff *et al.*, 2001; Webb *et al.*, 2001). The magnitude of fluorescence detected, and hence the bacterial cell population recovered during single species culture without treatment, showed the growth and fitness capability of fluorescent bacteria while fluorescing. These results suggest that expression of the red and green fluorescent proteins does not negatively affect the growth fitness and viability of the two fluorescently labelled species, consistent with published reports (Burcham *et al.*, 2016; Vickerman *et al.*, 2015; Pereira *et al.*, 2010; Barnes *et al.*, 2008; Maksimow *et al.*, 2002; Webb *et al.*, 2001).

The use of fluorescent spectrophotometry allowed real-time observation of the phage–treatment interaction. The findings of this study show that phage treatment effects can also be evaluated through fluorescence detection. There was a strong correspondence between the fluorescence detection and the bacterial population count result. For both the mCherry *S. aureus* KUB7 and GFP PAO1, the fluorescence signals continued to accumulate for no treatment, unaffected by the treatment, or negative control groups. Fluorescence signal magnitude of mCherry or GFP is reduced significantly when the mixed-species culture is treated with each phage cocktail alone.

The change in the fluorescence signal in non-target species of phage cocktail treated wells resembles the fluorescence signal pattern observed during single-species culture without treatment. The higher green fluorescent detected in *S. aureus* phage-treated mixed-species culture than in GFP PAO1 single-species PBS-treated culture might be attributed to the enhanced growth *P. aeruginosa* population because of getting more nutrient such as iron supply from the dead *S. aureus* cells (Mashburn *et al.*, 2005). The fluorescence magnitude and bacterial cell population obtained during AB-SA01+AB-PAO1 and gentamicin treatment were similar; the fluorescence records are low, the fluorescence graphs are overlapped and remained at their lower levels throughout the experiment period, and bacterial population recovered at the end of the experiment is minimal or none.

The magnitude of fluorescence detected during untreated mixed-species culture was lower compared to untreated single-species cultures, which might be attributable to competition between the two bacterial species (Armbruster *et al.*, 2016; Baldan *et al.*, 2014; Michelsen *et al.*, 2014). All fluorescence detection results strongly associate with the respective colony count results. Continuous decrease or increase in fluorescence intensity with an associated decrease or increase in CFU, respectively, was consistently observed throughout the experiment. The reductions in bacterial cell count were associated with the susceptibility of each bacterial isolates to the specific phage cocktail and its component phages during spot test as it can be seen in Table 11 in the appendices. The bacterial cell density observed during untreated fluorescent mixed-species culture was similar to the result obtained during the co-culture study of the two species, including clinical isolates. This finding is supported by previous reports that show the magnitude of fluorescence, absorbance, and colony count results are supplementary to one another in showing bacterial growth (Kong *et al.*, 2016; Russo *et al.*, 2015).

The magnitudes of the fluorescence of mixed-species cultures treated with AB-SA01+AB-PAO1 and gentamicin were low but still higher than only AB-SA01 or AB-PA01 treated samples for each bacterial species. Nevertheless, this fluorescence magnitude difference was not significant. The time-dependent increase in the magnitude of fluorescence in untreated cultures and cultures treated with the phage cocktail of the other species is assumed to be consistent with the increase in that unaffected bacterial species population. While the fluorescence values of the untreated samples increase with time, it decreases for treated samples, which is consistent with efficient bacterial cell lysis by the host-specific phage cocktail, leading to a reduction in fluorescence. In this study, the decrease or loss of fluorescence corresponded with a decrease in the number of cells of each species, which is consistent with previous data comparing fluorescence detection with a colony count result (Webb *et al.*, 2001).

In mixed-species planktonic culture, infection with each phage cocktail alone caused a statistically significant decline of each bacterial host population ($p < 0.001$). Infection with the combination of the two phage cocktails (AB-SA01+AB-PA01) also resulted in a statistically significant decrease in cell density of both bacterial species ($p < 0.001$). These findings are similar to a study that showed planktonic *E. coli* grown in co-culture with *Salmonella* enterica did not survive because of the combined effect of infection from the lytic *E. coli* phage and competition from *Salmonella* enterica (Harcombe and Bull, 2005).

The effect of both phage cocktails separately and in combination was higher in planktonic culture than in biofilms, which agrees with previous reports (Fong *et al.*, 2017; Alves *et al.*, 2016; Mendes *et al.*, 2014; Kay *et al.*, 2011). Possible explanations include: the complex EPS may reduce efficacy of phages because of entrapment of phage particles in the biofilm matrix (Kay *et al.*, 2011), reduced multiplication of phages due to the large proportion of

metabolically inactive host cells (Harper *et al.*, 2014), and shedding of phage receptors from the host bacteria (Labrie *et al.*, 2010; Teplitski and Ritchie, 2009). This observation is consistent with the previous report that showed the application of phages phiIPLA-RODI, phiIPLA-C1C, and the combination of the two phages in the planktonic phase is more efficient than that in biofilm phase during *S. aureus* IPLA16 and *S. epidermidis* LO5081 mixed-species cultures (Gutierrez *et al.*, 2015). Tetracycline treatment in biofilms shows superior bacterial population reduction, on both bacterial species, compared to the phage cocktail formulations used in this study. Overall, the reduction of bacterial cell population during planktonic culture and biofilm treatment due to a specific phage cocktail or the combination of the two phage cocktails was significant.

Despite the current increasing interest in the role of biofilm in DFU infections, interspecies interactions within mixed-species biofilms are still poorly elucidated (Hoffman *et al.*, 2006). Our results are consistent with findings that demonstrate *S. aureus* growth and biofilm formation is affected in the presence of *P. aeruginosa* (Qazi *et al.*, 2006). When we compare the population of the two bacterial species under no treatment conditions, the population of *S. aureus* bacteria as measured by colony count was less by 0.7 $\log_{10}$ (CFU/ml) and 1.3 $\log_{10}$ (CFU/ml) in planktonic and biofilm states, respectively, compared to *P. aeruginosa* population. Suppression of *S. aureus* growth by *P. aeruginosa* might have occurred due to nutrient depletion, or production of specific metabolic inhibitors, or lytic enzymes by the latter bacteria (Machan *et al.*, 1991).

Several studies demonstrated an inhibitory effect of *P. aeruginosa* on *S. aureus* strains, including the highly virulent ones *in vitro* (Baldan *et al.*, 2014; Pastar *et al.*, 2013; Yang *et al.*, 2011; Qin *et al.*, 2009). In apparent contradiction to our results, a study that applied *P. aeruginosa* onto 5 day-old *S. aureus* biofilm, with 1:250 *P. aeruginosa* to *S. aureus* ratio,

showed stable co-existence between the two species (Woods *et al.*, 2018). The contradiction between the two co-existence study findings might be due to differences in mixed-species biofilm development methods, bacterial strain differences, age of the biofilms, and bacteria population ratio used.

In this study, phage control of mixed-species biofilms was investigated by two approaches, using phage cocktails specific for either of the bacterial species and a combination of the two phage cocktails, to determine the extent of population reduction. In the presence of 5% BSA and with 1:3 initial concentration of *P. aeruginosa*-to-*S. aureus,* both species grew well in biofilms. In mixed-species biofilms, treatment with a combination of AB-SA01 and AB-PA01 phage cocktails decreased the cell populations of both species. Compared to planktonic culture, the mixed-species biofilm cultures maintained higher numbers of each species in the presence of their specific phage cocktail or the combination of the two phage cocktails. Phage predation and bacterial competition might have a deleterious effect on planktonic populations, but the biofilm might have provided a sheltering mechanism whereby some bacteria can better persist despite the effects of phages and competition (Kay *et al.*, 2011). Studies have shown that phages can effectively reduce bacterial population in biofilms (Gonzalez *et al.*, 2017; Motlagh *et al.*, 2016; Gutierrez *et al.*, 2015; Parasion *et al.*, 2014; Sillankorva *et al.*, 2010).

When the mixed-species biofilms were treated with AB-SA01, AB-PA01, or AB-SA01+AB-PA01, the population of *S. aureus* and *P. aeruginosa* were lower than those in the non-treated control groups. These findings agree with the study findings that when *E. co*li-specific phages were added to an *E. coli-S.* Typhimurium mixed culture, *E. coli* became extinct or was maintained at a lower population density than without phage application (Harcombe and Bull, 2005). The present study provides evidence that a phage cocktail applied to mixed-species

biofilms can effectively reach and reduce the host cell population. The current study results differ from a previous observation (Tait *et al.*, 2002) in that it did not show that the presence of non-susceptible bacteria in mixed-species biofilms protected susceptible bacteria from being attacked by the specific phage. Because significant host bacterial cell reduction was obtained in this study not only with the combination of the two phage cocktails but also with each phage cocktail alone, the presence of bacterial protection from the non-susceptible host cannot be assumed as indicated by a report (Sillankorva *et al.*, 2010).

Mixed-species biofilms are complex microbial and extracellular matrix combinations in which the physiological state of bacterial host cells and the configuration of phage receptors play vital roles in the efficacy of phages (Gutierrez *et al.*, 2015; Sutherland *et al.*, 2004). This study shows that phage cocktails can potentially control mixed-species biofilms. Moreover, it demonstrates that despite the presence of non-susceptible hosts, phage cocktails can successfully lyse their host bacterium in a multispecies biofilm.

In conclusion, these findings suggest that the use of phage cocktails in mixed-species infection, with bacteria in planktonic or biofilm state, could provide practical alternative or adjunct to antibiotics in the settings of DFU infections. During the evaluation of phage cocktails treatment effect on fluorescent mixed-species culture, the fluorescence values increased for untreated samples, and decreased for treated samples, over time. This finding is consistent with efficient bacterial cell lysis by the phage cocktails in mixed-species culture - the decrease or loss of fluorescence associated with the decrease or absence of bacterial cells of each sample. The lytic efficacy of phage cocktails, both independently and in combination, was higher in planktonic cultures than in biofilms. The less efficacy of phage cocktails in biofilms could be because dead cells resulting from phage attack might facilitate the survival of remaining cells, or the reduced metabolism of bacterial cells in biofilms might poorly

support the proliferation of phages. This study confirms the lytic efficacy of AB-SA01, AB-PA01, and their combination on their hosts in mixed-species planktonic and biofilm states.

Chapter 5

Efficacy of Phage Cocktail Therapy in Diabetic Mouse Wound Infections Caused by Multidrug Resistant *Staphylococcus aureus*

Chapter 5: Efficacy of Phage Cocktail Therapy in Diabetic Mouse Wound Infections Caused by Multidrug-Resistant *Staphylococcus aureus*

Abstract

The diabetic foot ulcer (DFU) is a severe complication of diabetes mellitus. Antibiotic-resistant *Staphylococcus aureus* is frequently isolated from DFU infections. Phages represent an alternative or adjunct to antibiotic therapy. Here, the efficacy of AB-SA01, a cocktail of three *S. aureus Myoviridae* phages, made to cGMP standards and which has undergone two phase I trials, in the treatment of antibiotic-resistant *S. aureus* infections using a diabetic mouse model of deep skin wounds is described. Bilateral 6 mm excisional wounds, inflicted on the dorsum of Balb/c mice that were rendered diabetic by injecting streptozotocin, were infected with 6.7 $\log_{10}$ CFU of multidrug-resistant (MDR) *S. aureus*. For 5 days, infections were treated topically with AB-SA01, and control groups received topical saline or topical saline plus intraperitoneal (IP) vancomycin. Bacterial load and wound healing parameters were used to assess treatment efficacy. Wounds of saline-treated mice showed no healing, but expanded and became inflamed, ulcerated, and suppurating. In contrast, AB-SA01 treatment decreased the bacterial load with efficacy similar or superior to vancomycin treatment. In phage-treated mice, wound healing was similar or superior to vancomycin treatment. No adverse effect related to the application of phages was observed. The findings of this study suggest that topical phage treatment may be useful in treating antibiotic-resistant *S. aureus* DFU infections.

5.1 Introduction

5.1.1 Diabetic foot ulcers

DFU is a frequently encountered complication, and represent the leading cause of health facility admission, among diabetic patients (Thurber *et al.*, 2017). Complications of DFUs are multifactorial in origin and costly (Thurber *et al.*, 2017). The global burden of DFU is rising, affecting up to 26.1 million people each year (Armstrong *et al.*, 2017). DFU is the precipitating cause for nearly 90% of limb amputations among persons with diabetes (Lavery *et al.*, 2007). Worldwide, 6.3% of persons living with diabetes are affected by DFU, and the lifetime incidence of a foot ulcer among persons with diabetes is estimated at 19% to 34% (Armstrong *et al.*, 2017). The 5-year mortality rate following foot amputation due to DFUs is up to 74% (Robbins *et al.*, 2008).

DFU management is costly because it may involve imaging studies, vascular surgery, wound dressing for lengthy periods, debridement, antibiotic therapy, and management of metabolic abnormalities (Richard *et al.*, 2011; Lipsky *et al.*, 2004). In the USA, the annual cost of DFU management is projected at an additional $9-13 billion over the cost of diabetes itself (Rice *et al.*, 2013). In England, the cost of DFU management in 2014-2015 was estimated at between £837 million and £962 million (Kerr *et al.*, 2019).

5.1.2 Antibiotic-resistant *S. aureus* in DFU

Most DFU infections are polymicrobial with bacterial species ranging from aerobic Gram-positive cocci to anaerobic Gram-negative bacilli (Mendes *et al.*, 2012b). *S. aureus* colonizes the skin and persists in the nares; nasal carriage is associated with an increased risk of clinical infection (Soge *et al.*, 2016). It is a virulent pathogen frequently isolated from DFU (Reveles *et al.*, 2016; Messad *et al.*, 2015; Rich and Lee, 2005; Lipsky *et al.*, 2004). Between 40% -

50% of *S. aureus* isolates identified from DFUs are MRSA, for which there are limited antimicrobial treatment options (Ding *et al.*, 2012). A recent Australian study found that 43.5% of DFUs were infected with *S. aureus*, and nearly half were MRSA (Commons *et al.*, 2015). *S. aureus* is a serious concern since it is becoming MDR to the most commonly used antibiotics (Gupta and Prasad, 2011).

The empirical antibiotic treatment for DFU infection includes an antibiotic against *S. aureus.* Thus, there is a high risk of emerging resistance strains (Barwell *et al.*, 2017; Bader, 2008). Hence, antibiotic-resistance is a major obstacle in treating infections caused by *S. aureus* (Uçkay *et al.*, 2015; Lipsky *et al.*, 2004). Furthermore, treatment may be ineffective because of insufficient antibiotic concentration at the site of infection because of deficient vascularization (Lipsky and Hoey, 2009). Hence, this pathogen affects the provision of empirical antibiotic therapy for DFU (Uçkay *et al.*, 2015; Lipsky *et al.*, 2004).

5.1.3 Phage therapy in DFU

Before the advent of antibiotics in the 1940s, phages were widely used in the USA and Europe. However, due to the development of many antimicrobials, lack of sufficient understanding about the biology of phages, inconsistent clinical trial results, and challenging regulatory environment (Kutter *et al.*, 2015; Summers, 2012; Monk *et al.*, 2010), phage treatment was excluded from western medicine, while it has continued to be practiced in eastern Europe for >100 years to treat bacterial infections (Summers, 2012; Sulakvelidze, 2005). Lytic phages kill their bacterial host by lysis (bursting the infected bacterial cell to release progeny phages) (Young, 1992a). The process of phage infection and subsequent self-replication in bacteria offers advantages over antibiotics: phages amplify themselves at the infection site provided there are sensitive bacterial hosts (Wittebole *et al.*, 2014). Phages are specific for the host bacteria species, an advantage over broadly active antimicrobials, as

phages are not expected to disrupt a patients' normal microflora. Phages lyse biofilm forms of bacteria, such as those typically found in infected DFU (Wittebole *et al.*, 2014; Donlan, 2009).

There is much evidence that phage use is safe (Brüssow, 2012; Pirnay *et al.*, 2011), and its extensive Russian and Georgian use has few adverse event reports (Brüssow, 2012). Phages are the most common biological entities in the biosphere, and in the gut, they outnumber bacteria (Reyes *et al.*, 2012; Edwards *et al.*, 2019). Phages are common in many environments; for instance, seawater has about 10^7 phage particles/ml (Ortmann and Suttle, 2005). In 2006, the US Food and Drug Administration (FDA) gave GRAS ("Generally Regarded As Safe") status to food additive phage product Listex, against *Listeria monocytogenes* (Sharma, 2013); many phage products in this industry now have such status. There is no evidence in the literature that rapid bacterial lysis triggers harmful inflammatory responses to released bacterial contents.

The US Kutter group used Eliava Institute, Georgia, *S. aureus* phage Sb-1 to treat recalcitrant DFU infections (Fish *et al.*, 2016). In this 5-patient study, ulcers were all single-toe lesions, *S. aureus*-infected, unresponsive to standard antibiotics, with exposed, infected bone, and amputation was likely. Phages were applied for 2 days, then 5 days of standard dressings, repeated weekly. In each case, full wound closure was achieved, and the toes were saved. The product used was sequenced, purified, and approved for use in Georgia, but not made to cGMP quality, hindering the uptake of this product to western medicine. Further studies with a product that meets cGMP regulations would support the uptake of this treatment to broader western medicine.

Topical application of lytic phage treatment accompanied by appropriate wound dressing could effectively decrease bacterial load and improve wound healing in antibiotic-resistant

infections (Rhoads *et al.*, 2009). To date, experimental evidence demonstrating the efficacy of phages in chronic DFU infections is scarce. This study sought to investigate the antibiotic activity and wound-healing potential of a topically administered phage cocktail. A cocktail of 3 *S. aureus* phages, designated AB-SA01, which has undergone two phase I trials and made to cGMP standard (Lehman *et al.*, 2019), was used in this study. The objective of this work was to determine the efficacy of this cGMP phage product in treating *S. aureus* infection in a diabetic mouse model.

5.1.4 Mouse skin wound model

To mimic the impaired wound healing in diabetes, animal models, especially mice, have been utilized (de Mayo *et al.*, 2017; Turner *et al.*, 2017). Mice wound models are most commonly used because of low cost, ease of management, and the ability to mimic key aspects of human wound healing (Park *et al.*, 2014). Excisional wound models in mice have been used to evaluate the efficacy of topically applied antibiotic agents (Davidson *et al.*, 2013). Unlike the re-epithelization and granulation process of human wound healing, wound healing in mice is through skin constriction (Dunn *et al.*, 2013). To mimic human wound healing in mice, a silicon sheet splint model that prevents excessive wound constriction was used (Park *et al.*, 2014; Lindblad, 2008). Due to the encouraging efficacy of AB-SA01 on single- and mixed-species planktonic cells and biofilms, as discussed in chapters 2 and 4 of this thesis, the diabetic mice splint wound infection treatment study reported herein was undertaken. In this chapter, the MDR *S. aureus* bacterial load reduction and wound healing in diabetic mice wound following AB-SA01 treatment is described.

5.2 Materials and methods

5.2.1 Bacterial isolates

Nine *S. aureus*, eight clinical isolates, and one laboratory strain, identified using standard microbiology methods and confirmed by MALDI-TOF MS, were examined as candidate organisms for this work. The antibiotic susceptibility profile of the isolates was determined using VITEK® 2. Furthermore, their susceptibility for phage cocktail AB-SA01, both in planktonic and biofilm states, was examined using methods detailed in chapter 2 of this thesis. All clinical isolates were MRSA, and four of them were also MDR. All isolates were susceptible to AB-SA01 and its three components phages in planktonic and AB-SA01 in biofilm cultures *in vitro.*

5.2.2 Phage cocktail

AmpliPhi Biosciences Corporation provided phage cocktail AB-SA01. AB-SA01 is a combination of *Myoviridae* phages designated J-Sa 36, Sa 83, and Sa 87. The mean titer was 9.3 $\log_{10}$ (PFU/ml) for J-Sa 36 and Sa 83, 9 $\log_{10}$ (PFU/ml) for Sa 87, and 9.1 $\log_{10}$ (PFU/ml) for AB-SA01. The titer was evaluated on *S. aureus* laboratory strains RN4220 and SA6538 using plaque assay (Mirzaei and Nilsson, 2015; Merabishvili *et al.*, 2009). All component phages were fully sequenced by AmpliPhi Biosciences to confirm that they are free from antibiotic resistance or bacterial virulence genes (Lehman *et al.*, 2019).

5.2.3 Mice management

Female Balb/c mice were obtained from the Animal Resources Centre, Perth, Western Australia. Mice were free of obligate and opportunistic pathogens, including *S. aureus* https://www.arc.wa.gov.au/wp-content/uploads/2019/07/BALBc-nude-stock-health-report-May-

2019q.pdf. All experiments were approved by the Animal Welfare Committee, Flinders University/Southern Adelaide Local Health Network. Experimental design and reporting were carried out in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010) and were consistent with the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th edition, 2013. Mice were kept at the College of Medicine and Public Health Animal Facility at 22 ± 3 ^{O}C and 55 ± 5% humidity under 12:12 hour light-dark cycle in Tecniplast GM500 Mouse IVC Greenline cages (Tecniplast Australia Pty Ltd, New South Wales, Australia) using corncob bedding. Mice were kept in *S. aureus* free housing conditions and provided water and a meat-free rodent maintenance diet (Glen Forrest Stockfeeders, Western Australia, Australia) *ad libitum.*

Mice were weighed 2-3 times each week and monitored for hunching, ruffled coat, lethargy, cold to touch, crinkling of skin, sunken eyes, and rapid or labored breathing at least once daily. Mice that showed over 15% weight loss or were critically ill were euthanized. Vancomycin (Sigma-Aldrich Corporation, New South Wales, Australia) was assessed for possible toxicity on six mice in a separate pilot study at 150 mg/kg dose rate, twice daily IP, for five consecutive days as described (Gibson *et al.*, 2007); these mice did not display any apparent adverse reaction. At the end of the experiment, mice were euthanized using 3% isoflurane, followed by cervical dislocation. Post-mortem examination of the external surfaces and visceral organs was conducted immediately following an established procedure (Parkinson *et al.*, 2011).

5.2.4 Induction of diabetes in mice

After a week of acclimatization, a total of 48 female 8-week-old Balb/c mice, housed in groups of 5, received STZ injection (Sigma-Aldrich Corporation, New South Wales, Australia) following an established protocol (Ito *et al.*, 2001; Paik *et al.*, 1980). STZ is a

naturally occurring alkylating antineoplastic agent that is toxic to pancreatic insulin-producing cells and which is used to treat pancreas islet cell carcinoma and to induce diabetes mellitus in laboratory rodents (Deeds *et al.*, 2011). After 4 hours of fasting, each mouse received an IP injection of 50 mg/kg freshly dissolved STZ in 0.05M citrate buffer pH 4.5 once daily for five consecutive days to avoid the acute toxicity of STZ. Balb/c mice are among the least susceptible to STZ toxicity (Hayashi *et al.*, 2006; Le May *et al.*, 2006; Bock *et al.*, 2005) and STZ-diabetic induced nephropathy (Hayashi *et al.*, 2006; Paik *et al.*, 1980).

Non-fasting blood glucose levels (BGLs) were checked every 2 - 3 days by tail vein bleeding after applying 3% lidocaine/prilocaine anesthetic cream. BGLs were measured using an Accu-Chek Performa (Roche Diabetes Care Australia, New South Wales, Australia) blood glucose meter. Mice were restrained using a modified tube, and the tail was cleaned with three alternating scrubs of 70% alcohol. Following a recommended procedure (Yano *et al.*, 2012), the distal end of the prominent tail vain was pricked using a 27-gauge needle, and blood was collected by gentle milking of the tail. The first drop of blood was touched by the tip of a test strip inserted into the digital blood glucose meter, and the reading was recorded. Blood flow was stopped by applying pressure on the pricked vein with sterile gauze. Blood glucose measurement using the blood glucose meter is a quick method to assess non-fasting BGL and needs only a drop of blood compared to the clinical chemistry method (Grant *et al.*, 2012).

Mice with non-fasting BGL < 13.9, between 13.9 and 22.2, and ≥ 22.2 mmol/L on at least two different days were categorized as normal glycaemic, moderately hyperglycaemic, or severely hyperglycaemic status, respectively (Grant *et al.*, 2012). Mice were supplied with wet food and water *ad libitum.* Mice with no clinical signs were transferred to a group maintenance monitoring record and monitored for 4 weeks. Mice that showed any clinical

signs were monitored twice daily. Diabetic mice were randomly assigned to one of the following groups: uninfected phage-, infected saline-, infected phage-, and infected vancomycin-treated.

5.2.5 Insulin treatment of diabetic mice

Diabetic mice were treated with 1.0 international unit (IU) NovoMix® 30 (Novo Nordisk, New South Wales, Australia) insulin daily subcutaneously to ameliorate the hyperglycaemic effects of diabetes and maintain body weight (Guo *et al.*, 2015). NovoMix® 30 is a pre-mixed neutral suspension consisting of rapid-acting (30%) and longer-acting (70%) protamine insulin (Thorisdottir *et al.*, 2009). The insulin treatment was administered between 9:00 am – 10:00 am daily.

5.2.6 Selection of *S. aureus* isolates for mice skin wound infections

In a pilot experiment, eight clinical and one laboratory *S. aureus* isolates were examined for their capability to establish skin infection on mice. Bilateral excisional wounds at the back of the mice 30-50 mm from the base of the skull and 10 mm from the midline were inflicted on each mouse as described (Holtfreter *et al.*, 2013; Dunn *et al.*, 2013; Mendes *et al.*, 2013). This area was chosen to hinder the grooming of the wound by the mouse itself. Each isolate was inoculated into two mice, with two PBS-inoculated mice used as negative controls. The results of the pilot experiment are below.

5.2.7 Preparation of bacteria for infection

An isolated colony of each *S. aureus* isolate was taken from 16 hours culture on MSA plates and grown in 3 ml TSB (Thermo Fisher Scientific, South Australia, Australia) overnight. One milliliter of overnight broth culture was adjusted to OD_{600} of 0.7 using an SP-830+ Metertech spectrophotometer (Adelab, South Australia, Australia) that corresponds to 8.3 $\log_{10}$

(CFU/ml), through dilution by sterile physiological saline. Then, the broth culture was centrifuged at 3000 rpm, and pelleted cells were washed twice and resuspended with 1 ml PBS and ready for inoculation into the wound.

Bacterial density determination, in CFU/ml and then transformed to $\log_{10}$, was carried out according to an established protocol (O'Toole, 2016). Fifty microliters of bacterial suspension were serially diluted into 450 µl of TSB. One hundred microliters bacterial suspension was mixed with 3 ml tryptic soya soft agar, spread on pre-warmed at 37 ^{o}C MSA plates in triplicates, and incubated for at least 24 hours. Colony count was performed on a plate containing 30-300 colonies. The total bacterial population was calculated by using the formula B = N/d where B = number of bacteria; N = average number of colonies counted on three plates; d = dilution factor (Mendes *et al.*, 2013).

5.2.8 Infection of mouse wound

Fifty microliters of bacterial suspension in PBS, containing an average 7.1 $\log_{10}$ CFU, was applied to each mouse wound immediately after wound infliction. An equal volume of sterile PBS was inoculated into wounds of control mice. The wounds were then immediately overlaid with Opsite (Independence Australia, South Australia), which is a semi-occlusive waterproof film that adheres to the mouse skin. The infection was left for 3 days. On the 3rd and 10th days of infection, swab samples were taken by scrubbing the surface of each wound using sterile swabs to assess the establishment of infection. Each swab was rotated three times clockwise with enough pressure to produce a small amount of exudate from the wound and inserted into a separate tube of 3 ml TSB.

5.2.9 Quantification of bacteria in wound swabs

Tubes containing swabs were vortexed for 5 seconds, and a 100 µl aliquot was used for serial dilutions. Quantification was performed using the 10-fold serial dilution method. One hundred microliters of each dilution were mixed into 3 ml tryptic soya soft agar warmed at 42 ^{o}C and immediately plated onto prewarmed at 37 ^{o}C MSA plates in triplicate. Colony count was performed following the standard procedure after 24 hours incubation (O'Toole and Wong, 2016). To evaluate the bacterial load of the wound, the quantity of CFU was determined by taking the average of colony counted as described earlier (Mendes *et al.*, 2013; Capparelli *et al.*, 2007) and a wound with more than 5.0 $\log_{10}$ CFU on any of the days was considered critically infected (Levin and Antia, 2001).

5.2.10 Excisional wound infliction

Diabetic mice were conditioned with lemon-flavored paracetamol in drinking water at 1.34 mg/ml 3 days before wound infliction and provided for the entire experimental period. An established rodent wound infection model (Dunn *et al.*, 2013) was used. Mice were anesthetized using 3% isoflurane inhalation and maintained on 1.5% during surgery. Hair was removed on the dorsal skin using electric clippers and depilatory cream, and skin quickly sterilized using 70% ethanol. Mice were given a single 0.05 mg/kg buprenorphine injection subcutaneously just before wound infliction. Mice were injected with subcutaneous 170 µl PBS immediately before the surgery to prevent dehydration. Lubricating eye drops were applied during surgery to protect eyes.

A skin wound extending through the *Panniculus carnosus* muscle was inflicted, using a 6 mm sterile biopsy punch and fine scissors, as indicated above. The bilateral wounds were inflicted at 10 mm on either side of the midline and 30 mm from the base of the skull. Swab

samples were collected from each wound to assess any pre-existing *S. aureus*. A sterile 1 mm thick silicone splint with a 7 mm diameter circular hole at the center was applied over the wound and sutured onto the skin concentric with the wound using 5-0 nylon suture. Cyanoacrylate glue was used to fix the silicone sheet splint to the skin before suturing. The purpose of using silicone splint was to mimic the human wound healing process through avoiding contraction type of mice wound healing. All surgical procedures were performed in a clean surgery room using sterile instruments.

5.2.11 Preparation of an isolate for mouse wound infection and treatment experiment

Among the eight *S. aureus* clinical isolates that established infection, MDR *S. aureus* 63-2498 isolate, which was susceptible to AB-SA01 and vancomycin, and showed higher CFU than the other MDR isolates, was selected for diabetic mice excisional splint wound infection treatment experiment. It was identified using MALDI-TOF MS. MDR *S. aureus* 63-2498 was tested using VITEK® 2 system and demonstrated resistance to multiple antibiotics, including benzylpenicillin, oxacillin, ciprofloxacin, erythromycin, and clindamycin and susceptibility to vancomycin. A single colony of MDR *S. aureus* 63-2498 from MSA plate was taken and grown in 3 ml TSB overnight. One milliliter from the TSB overnight broth culture was taken and adjusted to an optical density of 0.7 at OD_{600} using an SP-830+ Metertech spectrophotometer that corresponds to 8.3 log_{10} (CFU/ml) through dilution with sterile PBS. The adjusted broth culture was centrifuged at 3000 rpm, and pelleted cells were washed twice and resuspended with 1 ml PBS. This suspension was used for inoculation into the wound.

Mice were randomly assigned into *S. aureus*-inoculated phage-treated (n = 8), *S. aureus*-inoculated vancomycin-treated (n = 6), *S. aureus*-inoculated PBS-treated (n = 7), and PBS-inoculated phage-treated (n= 8) groups. Each *S. aureus*-inoculated mice wounds were infected with 50 µl bacterial suspension containing 6.7 log_{10} CFU of MDR *S. aureus* 63-2498

isolate prepared as under preparation of bacteria for mice wound infection study using the established procedure (Mendes *et al.*, 2013). PBS-inoculated mice wounds received 50 µl of PBS. Suspensions were applied directly into wounds using sterile pipette tips, overlaid with gauze, and covered with Opsite. On days 3, 5, and 7 post-infection, the Opsite was removed, and swab samples were taken. Treatments were administered immediately post-sample collection.

5.2.12 Vancomycin treatment

A pilot experiment was carried out to evaluate the toxicity of vancomycin treatment on mice. Six mice without wound infection were injected vancomycin (Sigma-Aldrich Corporation, New South Wales, Australia) for five consecutive days through IP at 150 mg/kg twice a day. Mice were checked for 10 days for any clinical signs. After 10 days post-treatment, mice were euthanized, and gross pathological lesion examination was conducted. During the wound infection treatment, vancomycin was administered IP at 150 mg/kg twice daily for five consecutive days, from day 3 to day seven post-infection, as described (Gibson *et al.*, 2007). This dosage resembles human dosing (Takigawa *et al.*, 2017; Domínguez-Herrera *et al.*, 2016; Docobo-Pérez *et al.*, 2012; Wold and Turnipseed, 1981).

5.2.13 Phage treatment

Treatments were administered immediately after sample collection commencing on day 3. Gentle debridement was applied before administering the treatments, as described previously (Seth *et al.*, 2013). Gauze (10 x 10 mm) was soaked with 70 µl AB-SA01, equivalent to 7.9 $\log_{10}$ PFU or with 70 µl PBS solutions for control and vancomycin-treated mice, were applied to wounds and covered with Opsite, on days 3, 5, and 7.

5.2.14 Assessment of treatment effect

Wound size was measured in duplicate from different directions using a digital Vernier calliper by tracing the leading edge of epithelium within the wound (Shah *et al.*, 2013). The wound size on the excision day was defined as the original wound size. To assess the bacterial load, 100 µl of 10-fold serially diluted swab sample suspensions were mixed with 3 ml trypticase soy soft agar and cultured on MSA. After 24 hours incubation at 37 ^{o}C aerobically, colony count was performed on plates with 30 - 300 colonies as recommended (O'Toole, 2016). The bacterial population was calculated as above.

5.2.15 Data management and statistical analysis

Data were managed using Microsoft Excel. SPSS software (IBM SPSS Statistics version 25) was used for statistical analysis. Data were reported as the mean ± standard error of the mean and were compiled from at least three separate *in vitro* experiments or six mice per treatment group. A comparison of experimental groups was performed using one-way analysis of variance (two-tailed) or Student t-test. The association or independence of categorical variables was compared using Pearson´s chi-square test, $p < 0.05$ values were accepted as statistically significant.

5.3 Results

5.3.1 Maintenance and clinical monitoring of mice

Mice were assessed for behavioral and physical abnormalities. Presence or absence of hunching and ruffled coat, lack of movement on stimulation, coldness to touch, crinkling of skin, sunken eyes, rapid and labored breathing, deterioration of nest quality, and wound infection were checked during clinical monitoring. None of the mice manifested any of these signs during the infection and treatment period, except one mouse from the infected-untreated group that manifested hunching and ruffled coat for 3 days. In the first 2 weeks post-STZ injection, most of the mice became diabetics, manifested sustained hyperglycemia, and continued weight loss. Subjective observation of cage litter showed increased urine production requiring daily rather than every third-day litter changes, consistent with polyuria. Furthermore, water intake by diabetic mice was estimated to be double their intake pre-STZ injection intake, indicative of polydipsia.

5.3.2 Infection establishment of *S. aureus* isolates on mice wound

Mice were monitored twice daily for signs of fatigue, stress, aggressiveness, weight loss, and any form of shock syndrome. No apparent clinical sign of systemic infection was observed for the 10-days follow-up time. From 18 infected wounds during the pilot *S. aureus* infection establishment study, 17 developed purulent exudate and necrosis. The remaining wound was relatively clean. All infected wounds presented signs of inflammation, including erythema, warmth, swelling, and tenderness at the earlier times of the experiment. At the later stage, wounds showed purulent exudate, discolored granulation tissue, and foul odor. All the saline inoculated control wounds were clean. At the end of the experiment, the mean wound diameter of infected mice was 7.7 mm, whilst the uninfected control wounds were 3 mm.

5.3.3 Evaluation of bacterial load during infection establishment study

All swab samples of infected wounds yielded *S. aureus* isolates on MSA. The bacterial load of these samples ranged from 7.9 to 9.9 log_{10} (CFU/swab), as shown in Table 5.1. None of the control mice wounds yielded *S. aureus.*

Table 5. 1 Bacterial cell count of *S. aureus* from mice wound infection

ID	Antibiotic susceptibility	Log_{10} (CFU/swab)
63-5897	MRSA	9.1
63-2498	MRSA, MDR	9.4
63-7148	MRSA	9.7
60271003	MRSA	9.9
63-169	MRSA, MDR	8.5
63-6758	MRSA	9.6
63-7647	MRSA, MDR	8.9
46834854	MRSA, MDR	7.9
SA6538	Susceptible to all	8.6
Mean $\pm$ err		**9.1 $\pm$ 0.7**

5.3.4 Assessment of vancomycin toxicity in mice

During the vancomycin toxicity test, mice presented no visible clinical signs, and post-mortem examination did not show any abnormalities.

5.3.5 Induction of diabetes

Of 48 female Balb/c mice that received STZ, 28 (58.3%) and 12 (25%) developed non-fasting BGL $\geq$ 13.9 mmol/L within the first and second weeks of post-STZ administration, respectively. Of the remaining mice, three (6.3%) failed to develop diabetes, and five (10.4%) died before their diabetes status was determined. Of the diabetic mice, 21 (52.5%) developed severe hyperglycaemia, as defined above. While the mean body weight of mice before STZ administration was 18.9 ± 0 gram, the mean body weight of normal glycaemic, moderately

hyperglycaemic and severely hyperglycaemic mice groups were 19.3 ± 1.0, 17.8 ± 1.4, and 16.7 ± 1.4 gram, respectively, at the end of the diabetes maintenance period.

5.3.6 Effect of insulin treatment on hyperglycaemic condition and body weight of mice

Following the increased number of severely hyperglycaemic mice and continued weight loss, 1 IU NovoMix® 30 was administered subcutaneously for diabetic mice daily. There was no significant difference between BGL records of 1 week pre- and post-insulin treatment in both hyperglycaemic groups. After 2 weeks on insulin treatment, the moderately hyperglycaemic mice showed 8.7% mean BGL decrease while severely hyperglycaemic mice showed a 4.5% mean BGL increase. The mean BGL measured on random days showed no significant difference from the record before the insulin treatment was started for both diabetic mice groups ($p > 0.05$; 20.5 vs 21.1 mmol/L for severely hyperglycaemic and 17.3 vs 17.5 mmol/L for moderately hyperglycaemic groups), as shown in Table 5.2. In general, the effect of insulin treatment in ameliorating the higher BGL condition was not pronounced in any of the diabetic groups.

To assess the effect of insulin treatment on body weight gain, mice were weighed twice a week at 9:00 am in their holding house. In general, there was a steady average body weight increment in diabetic mice, similar to the non-diabetic mice. No statistically significant difference was observed in body weight gain between insulin-treated moderately and severely hyperglycaemic groups ($p > 0.05$; Table 5.2). Mice that failed to maintain body weight, before the commencement of the wound infection treatment study, were euthanised.

Table 5. 2 Effect of insulin on BGL, body weight, and mortality of mice. BGL is in mmol/L; mouse weights are in grams.

	Glycaemic status											
	Severely hyperglycaemic				**Moderately hyperglycaemic**				**Normal glycaemic**			
Insulin injection	**No of mice**	**BGL**	**Body Weight**	**Died/ Euth.**	**No of mice**	**BGL**	**Body Weight**	**Died/ Euth.**	**No of mice**	**BGL**	**Body Weight**	**Died/ Euth.**
Before	21	21.1 ± 4.0	15.2 ± 1.6	0	19	17.3 ± 3.1	16.0 ± 1.7	0	3	8.3 ± 0.9	18.2 ± 1.0	0
1 week after	21	23.9 ± 3.6	15.4 ± 1.7	0	19	18.0 ± 3.3	16.4 ± 1.8	0	3	7.7 ± 1.0	18.6 ± 0.9	0
2 weeks after	21	22.6 ± 4.2	16.1 ± 2.0	0	19	15.8 ± 4.6	17.1 ± 1.7	0	3	7.9 ± 1.0	19.0 ± 0.7	0
3 weeks after	21	---	17.5 ± 2.2	0	17	---	18.6 ± 1.6	2	3	---	19.7 ± 1.0	0
4 weeks after	19	---	18.5 ± 2.2	2	15	---	19.5 ± 1.7	2	3	---	20.5 ± 1.5	0
5 weeks after	18	---	19.1 ± 2.3	1	15	---	19.8 ± 1.6	0	3	---	20.6 ± 1.4	0
6 weeks after	18	---	---	0	15	---	---	0	3	---	---	0
7 weeks after	16	---	---	2	13	---	---	2	3	---	---	0
8 weeks after	15	---	---	1	12	---	---	1	3	---	---	0
Random days	15	20.5	---	0	12	17.5 ± 5.3	---	0	3	7.1 ± 0.4		0
At the end of the experiment	15			6	12			7	3			0

Euth. represents euthanised.

5.3.7 Result of splint excisional wound infliction

Twenty-nine diabetic mice in good health and body condition were selected for infection treatment using the splint excisional wound model experiment. A pair of splint excisional wounds were inflicted on each mouse, as illustrated in Figure 5.1, and mice were categorized under four treatment groups. Most of the silicone sheet splint stayed fixed on the skin of mice, but a few needed re-suturing. Both the adhesive and anchoring sutures kept the splint on the skin.

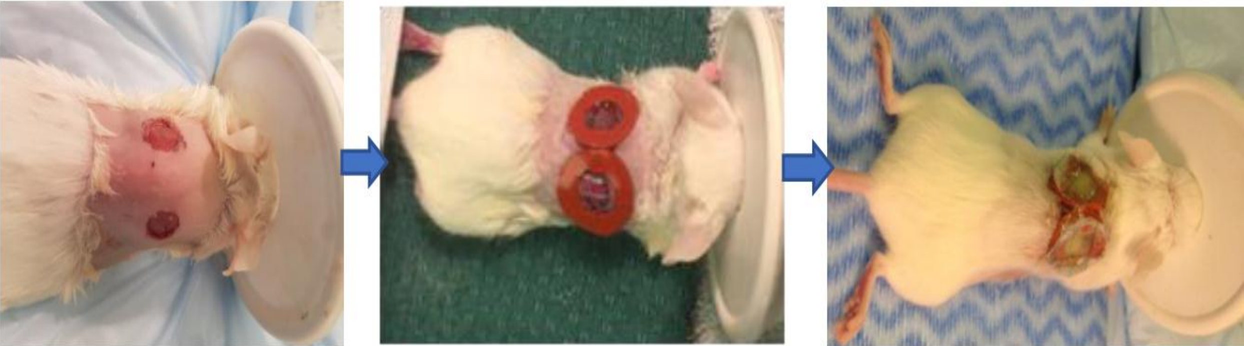

Figure 5. 1 Splint excisional wound infliction and wound splint silicone sheet application: shaved wound area and 6 mm diameter fresh wound (first figure), silicone sheet splint applied (second figure) and gauze and Opsite applied after infection is inoculated (third figure).

5.3.8 Result of inoculation of MDR *S. aureus* into diabetic mice splint wound

The establishment of MDR *S. aureus* in the diabetic mice splint wound was examined on day 3 of inoculation using swab samples. The colony count result demonstrated that all infected wounds yielded *S. aureus*. The mean bacterial cell count ranged from 7.0 - 9.0 $\log_{10}$ (CFU/swab). The non-infected control mice's wounds did not produce significant numbers of *S. aureus*.

5.3.9 Effect of AB-SA01 on MDR *S. aureus* infection in diabetic mice wounds

The bacteria count on days 3, 5, and 8 post-treatment showed a decrease in all phage- and vancomycin-treated mice. No *S. aureus* was detected from more than half (n = 5/8) of phage-treated wounds, and the mean bacterial load from this group was 1.1 log_{10} (CFU/swab) on day 8 of the treatment period. In contrast, 4.8 log_{10} (CFU/swab) mean *S. aureus* cells were detected in vancomycin-treated mice on day 8 of the treatment period, as shown in Figure 5.2 and Table 5.3.

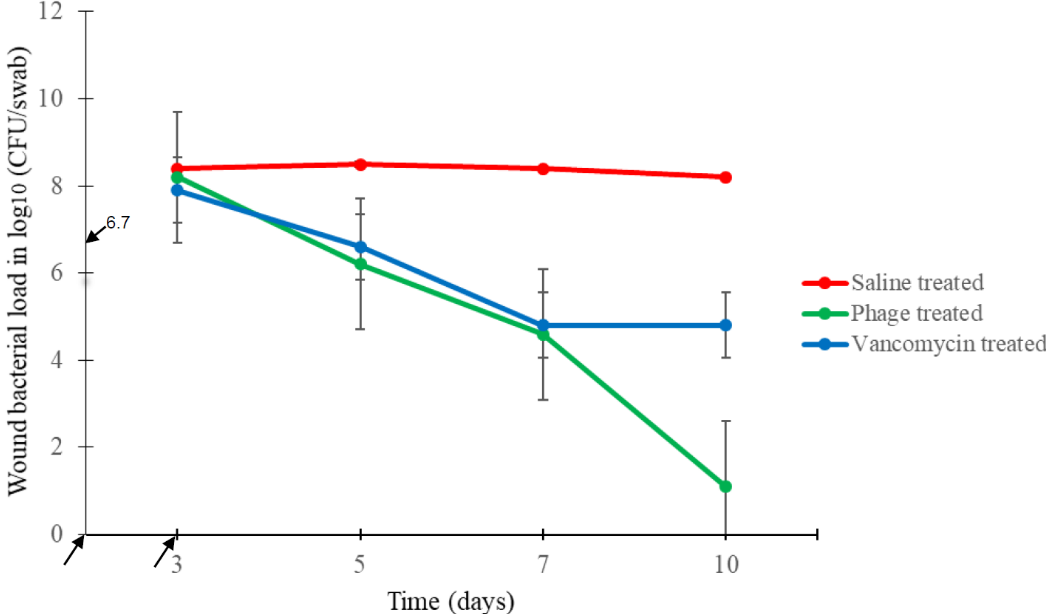

Figure 5. 2 Effect of phage treatment on MDR *S. aureus* bacterial load compared to control and IP vancomycin treatment. Saline-treated mice (control, red); AB-SA01 phage-treated mice (green); vancomycin-treated mice (blue). The arrows on the horizontal axis indicate the infection date (first arrow, day 0) and treatment start date (second arrow, day 3), respectively. Treatments were applied from day 3 to day 7, as detailed in the methodology part of this chapter. The arrow on the vertical axis indicates the infection dose.

A statistically significant bacterial load decrease ($p < 0.05$) was observed on day 3 of the phage cocktail and vancomycin treatment. A more pronounced bacterial cell population reduction ($p < 0.001$; Figure 5.2 and Table 5.2) was observed on day 5 of the treatment period for both phage-treated and vancomycin-treated groups. On day 8 of treatment, the bacterial load reduction due to phage cocktail treatment was statistically different from vancomycin treatment ($p < 0.05$; 1.1 vs 4.8 $\log_{10}$ (CFU/ml)). There was no significant number of *S. aureus* detected from uninfected phage-treated mice wounds throughout the experimental period.

Table 5. 3 Bacterial loads (in $\log_{10}$ CFU/swab) in diabetic mice wounds infected with MDR *S. aureus* isolate SA63-2498 and treated with phage cocktail AB-SA01 or controls.

Mouse ID	Treatment group	Day 0	Day 3	Day 5	Day 7	Day 10
4-NEM	PBS	0	8.99	8.36	8.51	8.57
4-1L		0	7.72	8.11	8.43	7.91
4-2R		0	8.34	8.85	8.38	8.18
5-NEM		0	7.00	8.34	8.41	7.62
5-1L		0	8.76	8.70	8.20	8.11
5-2R		0	6.97	7.73	8.51	8.53
10-RL		0	7.00	8.38	8.26	7.95
Mean ± err		0	8.42 ± 0.9	8.47 ± 0.4	8.39 ± 0.1	8.23 ± 0.3
7-1R	Phage	0	8.40	6.43	2.26	0.00
7-1L		0	8.40	6.18	5.72	2.00
7-RL		0	7.88	6.45	6.30	2.28
7-2R		0	8.56	6.26	6.63	0.00
9-NEM		1.1	8.38	6.28	2.48	4.34
9-1R		1.5	8.81	6.45	5.15	0.00
9-1L		0	8.41	5.80	5.72	0.00
9-RL		0	6.66	5.43	2.32	0.00
Mean ± err		0.3	8.19 ± 0.7	6.16 ± 0.4	4.57 ± 1.9	1.08 ± 1.6
6-1R	Vancomycin	0	8.64	6.18	5.15	7.04
6-RL		0	7.46	4.70	3.96	5.86
6-2R		0	8.04	7.99	4.75	6.75
8-NEM		0	7.84	6.49	5.58	1.04
8-1L		0	7.80	6.52	4.28	6.04
8-2R		0	7.72	7.92	4.96	2.11
Mean ± err		0	7.92 ± 0.4	6.63 ± 1.2	4.78 ± 0.6	4.81 ± 2.6

5.3.10 Effect of AB-SA01 on diabetic mice wound healing infected with MDR *S. aureus*

Infected phage- and vancomycin-treated mice wound showed a decrease in size during and after the course of treatment, leading to complete wound healing. All uninfected phage-treated wounds also decreased in size through time and had complete healing at the end of the experiment. In contrast, infected PBS-treated wounds increased in size, with examples illustrated in Figures 5.3 and 5.4. The mean wound diameters at the end of the experiment were 0.0, 0.2, 0.3, and 7.8 mm for infected vancomycin-, infected phage-, uninfected phage-, and infected PBS-treated groups, respectively.

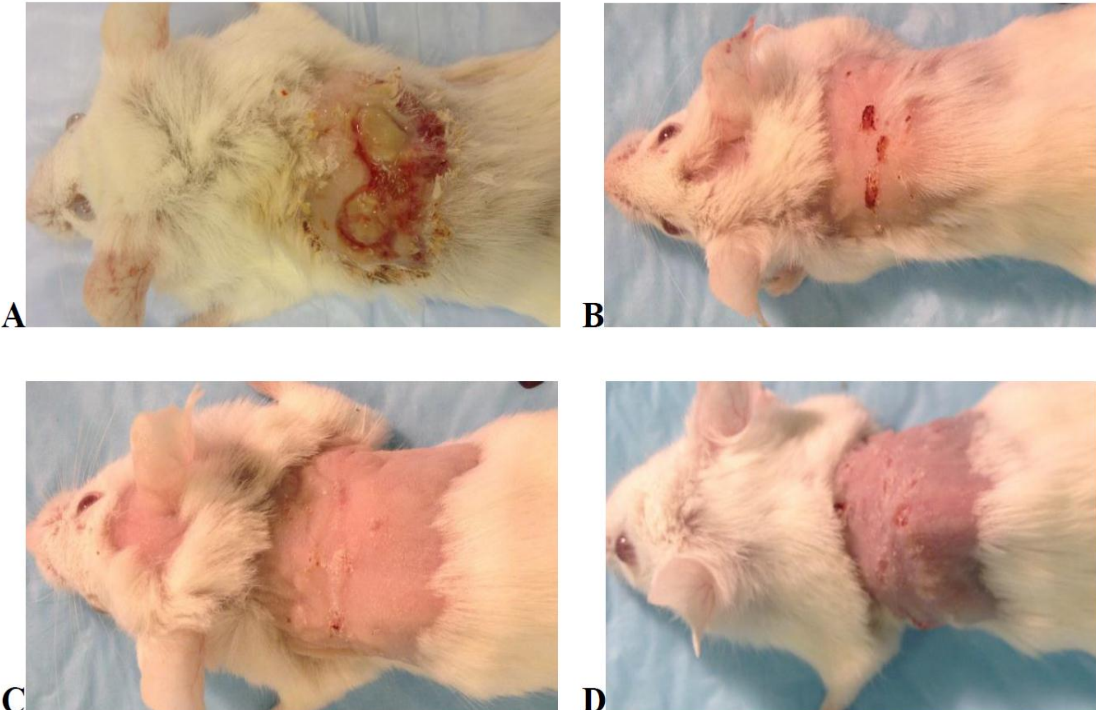

Figure 5. 3 Diabetic mice wound appearance at day 10: **A** infected PBS-treated, showing lack of healing and expansion of wounds; **B** uninfected phage-treated, **C** infected phage-treated, and **D** infected vancomycin-treated. Wounds in **B, C,** and **D** groups showed similar complete healing.

All infected PBS-treated wounds manifested non-healing ulcers characterised by purulent exudate, discoloured granulation tissue, and foul odour. Compared to the infected PBS-treated mice wound size, a statistically significant wound size decrease ($p < 0.001$) was found at the end of the experiment for infected phage- (7.8 vs 0.2 mm), uninfected phage- (7.8 vs 0.3 mm), and infected vancomycin-treated (7.8 vs 0.0 mm) mice. In contrast, the infected PBS-treated mice showed a statistically significant wound size increase at the end of the experiment compared to the original wound size ($p < 0.05$; 7.8 vs 6.0 mm). There was no statistically significant difference in wound healing progression among uninfected phage-, infected phage-, and infected vancomycin-treated groups ($p > 0.05$; Figure 5.4). Phage treatment caused no apparent adverse effects in mice in the absence or presence of its bacterial host.

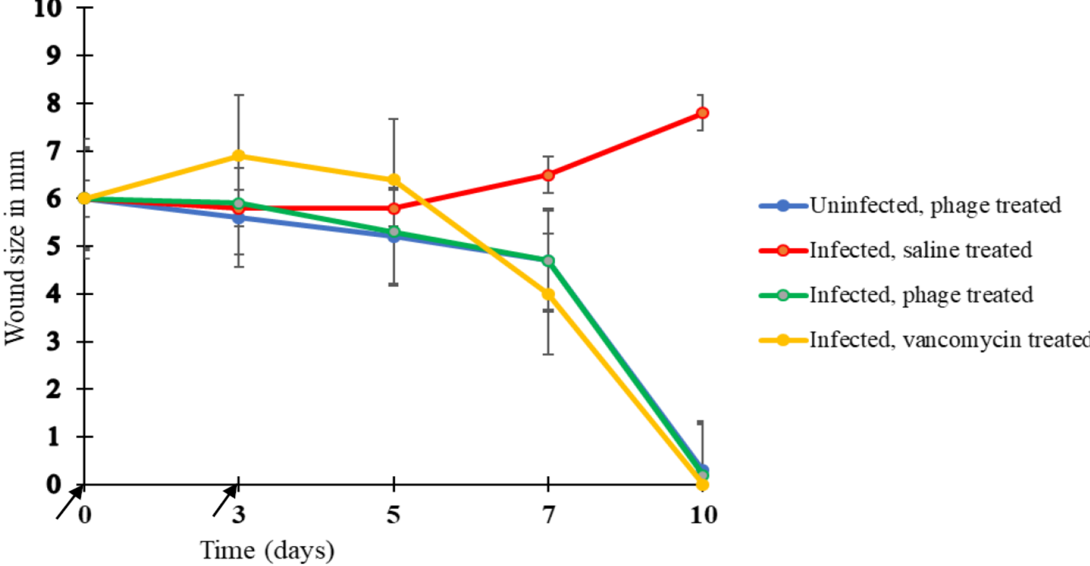

Figure 5. 4 Effect of phage AB-SA01 on wound healing. The arrows indicate the infection date (day 0), and the date treatment started (day 3). No healing was seen in infected PBS-treated mice (red). In contrast, complete wound healing was seen in uninfected phage-treated wounds (blue), and infected wounds treated with phage (green), and in the control group with infected vancomycin-treated wounds (yellow).

5.3.10 Clinical assessment of diabetic mice infected with MDR *S. aureus* and treated

Hunching, ruffled coat, lethargy, cold to touch, crinkling of skin, sunken eyes, and rapid or labored breathing were criteria used to check the health of mice. No mice manifested these signs of systemic infection during the wound infection and treatment periods. Non-healing wounds with purulent exudate, discolored granulation tissue, and foul odor were noted in all infected wounds before treatment for all treatment groups, and the entire period of the experiment in the infected untreated groups. No mortality was recorded associated with infection.

5.3.11 Mice mortality and post-mortem findings

The mortality rate of mice during the entire experimental period was 33.3% (n = 16/48). Euthanasia based on animal welfare contributed to half of the mortality records. Manifesting two or more clinical signs that are indicated in the maintenance and clinical monitoring sheets and body weight loss were reasons for euthanasia. Many of the mice were euthanized on the ground of unacceptable weight loss that is greater than 15% of their initial body weight. The mortality rate was similar among severely hyperglycaemic, moderately hyperglycaemic, and normal glycaemic mice. The highest mortality rate (68.8%, n = 11/16) was recorded between week 5 and 7 post-STZ injection. No mortality was observed during the excisional wound infection and treatment period. A post-mortem examination was conducted only on euthanized mice because of most mouse deaths overnight, and typically they were cannibalized. Post-mortem examinations did not show any evident pathological lesions other than the skin wounds. There was no food in the gastrointestinal tract, and little to no abdominal fat was observed.

5.4 Discussion

An earlier study demonstrated that *S. aureus* establishes infection on mice skin within 4 hours of inoculation (Kugelberg *et al.*, 2005). The present study found that MRSA and MDR *S. aureus* isolates were successful in establishing infection on mice skin in 3 days. Clinical signs observed during infection establishment experiments of this study include inflammation, suppurative exudate, and widened and deepened wound, similar to lesions due to *S. aureus* infections reported earlier (Malachowa *et al.*, 2013). The mean bacterial count results, 9.1 $\log_{10}$ (CFU/swab) showed the successful establishment of infection in mice skin by the clinical isolates of *S. aureus* collected from DFU patients, in agreement with earlier studies (Malachowa *et al.*, 2013; Kugelberg *et al.*, 2005).

Vancomycin is effective against most isolates of MRSA (Takigawa *et al.*, 2017). Vancomycin in mice has been non-toxic under standard dose, regimen, and duration (Domínguez-Herrera *et al.*, 2016; Gibson *et al.*, 2007). In this study, no evidence of toxicity was observed during the entire follow-up period, nor was any gross pathological lesion seen in any of the mice, consistent with similar studies (Domínguez-Herrera *et al.*, 2016; Docobo-Pérez *et al.*, 2012; Wold and Turnipseed, 1981).

Following the demonstration of the *in vitro* efficacy of phage cocktail AB-SA01 and its components on *S. aureus* isolates collected from DFU patients, as detailed in Chapter 2, the present study was initiated to examine the efficacy of AB-SA01 using a diabetic mouse wound infection model. The nongenetic diabetic mouse model was preferred because it mimics the late

stage of type 2 diabetes with a relative deficiency of insulin and a tendency for infections (Strowski *et al.*, 2004). Balb/c mice are among the least susceptible to STZ toxicity because they have relatively high pancreatic β-cell mass due to their large number of islets (Hayashi *et al.*, 2006; Bock *et al.*, 2005; Cardinal *et al.*, 1998). Moreover, female mice are relatively resistant to STZ toxicity compared to males because sex steroids may, to an extent, protect them from β-cell injury (Le May *et al.*, 2006). Besides, STZ-diabetic nephropathy is more pronounced in male mice compared to females (Hayashi *et al.*, 2006; Paik *et al.*, 1980). Hence, female Balb/c mice were selected for the current experiment. The induction of diabetes was started on 8-weeks-old mice because this age group is commonly considered appropriate in previous studies to the STZ-induced diabetes model (Dekel *et al.*, 2009; Ito *et al.*, 2001).

STZ is a broad-spectrum antibiotic extracted from *Streptomyces achromogenes* bacteria for the treatment of bacterial infections and carcinoma of pancreas islet cells (Deeds *et al.*, 2011). STZ has been commonly used for the induction of diabetes mellitus in laboratory rodents as it is toxic to β-cells of pancreatic islets responsible for insulin production. Injection of a low dose of STZ for five consecutive days has been recommended to develop progressive hyperglycaemia (Deeds *et al.*, 2011; Kume *et al.*, 2004; Paik *et al.*, 1980). The Animal Models of Diabetes Complications Consortium (AMDCC, http://www.amdcc.org) also recommends a low-dose model for STZ-induced diabetes. The result of this study agrees with these reports in terms of producing progressive hyperglycaemia and a large percentage of diabetic mice. This study found no significant difference between severely and moderately hyperglycaemic mice in terms of glycaemic control, weight gain, or survival rate through time.

The severe hyperglycaemia and substantial body weight loss in many mice observed in this study are consistent with a study that used a similar treatment regimen (Hayashi *et al.*, 2006). In an animal model of diabetes, it is essential to monitor the relationship between diabetes progression and loss of body weight to reduce excessive body weight loss (>10%) as well as mortality of experimental animals. In this study, about 33% of mice mortality was observed, which is higher than previous reports (Deeds *et al.*, 2011; Kume *et al.*, 2004; Paik *et al.*, 1980). Half of the loss of mice was due to STZ treatment-related morbidity and severe weight loss. No mortality occurred during the excisional wound infection and treatment period.

Phage cocktail AB-SA01 was well tolerated by the mice, as shown by the lack of clinical abnormalities such as a change in the well-being of mice or evidence of anaphylactic reactions due to harmful response to phages. This finding agrees with previous study results on the safety of phage therapy (Drilling *et al.*, 2017; Speck and Smithyman, 2016; Rhoads *et al.*, 2009; Capparelli *et al.*, 2007). Potential adverse effects such as toxic shock because of the rapid and considerable numbers of bacteria lysis and release of large quantities of toxins (Capparelli *et al.*, 2007) were not observed. The absence of adverse effects was expected as the AB-SA01 material used in this experiment is a well-characterized phage cocktail, produced under cGMP standards, and approved by that complies with the U.S. Food and Drug Administration (FDA) and the Australia's Therapeutic Goods Administration (TGA) to conduct clinical phase I trials and single-patient emergency treatment (Lehman *et al.*, 2019).

Skin wound healing in rodents involves skin contraction, a healing mechanism not seen in human wound healing, which occurs by secondary intention, granulation, and re-epithelialization (Fukui *et al.*, 2017; Park *et al.*, 2014; Mendes *et al.*, 2013). In this study, a splint wound model was employed to mimic human skin wound healing through avoiding contraction type of mice wound healing, as described (Dunn *et al.*, 2013).

In the current study, the therapeutic potential of AB-SA01, a cocktail of 3 *S. aureus Myoviridae* phages, was evaluated on diabetic mice to treat wounds infected with an MDR *S. aureus* clinical isolate resistant to multiple antibiotics, including those most commonly used to treat staphylococcal infections such as oxacillin, clindamycin, and trimethoprim-sulfamethoxazole. This phage application methodology is supported by data from a previous report (Seth *et al.*, 2013). AB-SA01 reduced bacterial load to low levels while the control mice were still infected with a high bacterial load. This finding is in line with studies that showed bacterial load reduction in mice infected with *S. aureus* (Capparelli *et al.*, 2007) and Wistar rats with *S. aureus* and *P. aeruginosa* (Mendes *et al.*, 2013). A significant bacterial load decrease was observed on day 3 post-AB-SA01 administration ($p < 0.05$), and it continued to decrease throughout the treatment period. The infection process in phage- and vancomycin-treated groups was controlled well by the end of the experiment compared to untreated control mice ($p < 0.001$).

The bacterial load in the vancomycin-treated group decreased at a similar trajectory to the phage-treated group during the treatment period but remained constant from the day treatment was completed onwards. The bacterial load reduction at the end of the experiment in the vancomycin-

treated mice was not as great, and significantly different from that in phage-treated mice ($p < 0.05$). The continued bacterial load reduction in phage-treated mice after the treatment was stopped demonstrates an advantage of phages over antibiotic treatment, because phages replicate in the presence of their bacterial host (Fu *et al.*, 2010; Payne *et al.*, 2000). Of 8 infected phage-treated mice, *S. aureus* had been eliminated from the wounds of 5 mice by the end of the experiment. In the three remaining mice with a detectable bacterial load, the bacterial population was minimal compared to that in PBS-treated mice wounds. In contrast to phage-treated mice, at the end of the experiment, *S. aureus* was still detected in all vancomycin-treated mice with a mean bacterial load of 4.8 $\log_{10}$ (CFU/swab). Four of the six vancomycin-treated mice had a residual bacterial burden of $\geq$ 5.9 $\log_{10}$ (CFU/swab) at the end of the experiment.

From the findings of this study, it is possible to conclude that phage treatment resulted in superior or equivalent efficacy to vancomycin. The data suggest that phages may be valuable antibiotic treatment adjuncts in this setting because they: a) may be used topically, b) show apparent excellent efficacy with a low number of treatments, which is compatible with standard wound care, c) show no local inflammatory reaction, d) may continue to work for an extended period post-administration, and e) have fewer documented adverse effects as compared to antibiotics such as vancomycin (Speck and Smithyman, 2016; Rhoads *et al.*, 2009).

The use of phage cocktails (instead of single phage) broadens the spectrum of activity of phage formulations and reduces the likelihood of development of phage-resistant bacteria (Chan *et al.*, 2013; Cairns and Payne, 2008). This could be because component phages complement each

other, possess different infection mechanisms, and may identify different receptors (Gu *et al.*, 2012). The three phage components in AB-SA01 were shown to broaden the spectrum of activity, complement each other, and reduce the frequency of bacterial resistance when combined (Lehman *et al.*, 2019).

Topical administration of phage cocktail AB-SA01 exhibited an effect similar to that observed with vancomycin in facilitating wound healing. The decrease in wound size associated well with the decrease in bacterial load. Wound size measurement showed a statistically significant difference between the treatment and control groups ($p < 0.001$). These observations are consistent with a previous report (Chhibber *et al.*, 2013). More than 85% of uninfected phage-, infected phage-, and infected vancomycin-treated mice wounds were completely closed, and hair regrowth was observed on some of the wounds. No statistically significant difference was observed in wound closure between these three treatment groups ($p > 0.05$).

In conclusion, the treatment of diabetic mice wounds infection caused by MDR *S. aureus* using topical application of phage cocktail AB-SA01 is effective with efficacy similar or superior to vancomycin treatment, as shown by bacterial load reduction and wound closure. The findings of this study show that phages represent a potentially effective topical treatment for diabetic ulcers infected with antibiotic-resistant pathogens.

Chapter 6

General Discussion

Chapter 6: General Discussion

6.1 Discussion

Diabetes mellitus is becoming a global epidemic. An estimated 463 million people are living with diabetes mellitus in 2019, which is expected to increase to 578 and 700 million people by 2030 and 2045, respectively (Saeedi *et al.*, 2019). DFU infection is a common, severe, costly, and disabling complication of diabetes mellitus and often associated with antibiotic-resistant polymicrobial infections (Alexiadou and Doupis, 2012). These infections are dominated by *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Lee *et al.*, 2017; Malik *et al.*, 2013; Ramakant *et al.*, 2011).

S. aureus and *P. aeruginosa* are frequently isolated together in DFU infections, with joint occurrence causing more severe disease than infection with each species alone (Bessa *et al.*, 2015; Trivedi *et al.*, 2014; Pastar *et al.*, 2013; Körber *et al.*, 2010; Hendricks *et al.*, 2001). Together, their biofilm-forming capability contributes to their resistance against antibiotics and the immune system (Steenackers *et al.*, 2016; Ramakant *et al.*, 2011; Dowd *et al.*, 2008; Gilbert *et al.*, 2002). The objective of this work was to evaluate the treatment efficacy of two phage cocktails and their components, AB-SA01 against *S. aureus* and AB-PA01 against *P. aeruginosa*, respectively. The efficacy evaluation was conducted using *in vitro* and *in vivo* experiments.

Infections from multi-, extensive-, and pan-drug resistant strains are common in various groups of bacteria (Courvalin, 2016). The increasing failure of antibiotics prompts the development of phages as alternative anti-infective agents. This study was conducted on laboratory strains, and clinical isolates of *S. aureus* and *P. aeruginosa* sampled during the study from DFU infection patients at diabetes clinics in Adelaide, South Australia. The two bacterial species were selected because they are the most common infectious agents in DFU patients based on epidemiological studies and treatment failure due to their high antibiotic-resistance characteristics (Saltoglu *et al.*, 2018; Kateel *et al.*, 2018) and because phages against these species are available. Chapters 2 and 3 of this thesis reported the results of identification and antibiotic susceptibility profiles of *S. aureus* and *P. aeruginosa* clinical isolates. These chapters also examined the lytic efficacy and host range of phage cocktails AB-SA01 and AB-PA01 and their components against laboratory and clinical isolate hosts in planktonic and biofilm phases.

Phages are specific antibacterial agents that are often effective against certain strains of a bacterial species (Abatangelo *et al.*, 2017; Pincus *et al.*, 2015; Kwiatek *et al.*, 2015; Balarjishvili *et al.*, 2015; Collins, 1955). Hence, developing phage formulations with broad-spectrum efficacy on most strains of significant DFU infection bacterial species is a challenge. It has been shown that for some bacterial species, phage cocktails can be formulated to lyse most strains of the target bacterial species (Lehman *et al.*, 2019; Fong *et al.*, 2017; Bernasconi *et al.*, 2017; Chan *et al.*, 2013). In this study, two phage cocktails: AB-SA01 against *S. aureus,* and AB-PA01 against *P. aeruginosa* are used. AB-PA01 contained four phage components: two *Myoviridae* and two *Podoviridae*. AB-SA01 contained two *Myoviridae* phages. An important aspect of bringing

phages to the clinic is that they are made to high standards. All component phages were exclusively lytic and did not encode bacterial virulence factor genes (Lehman *et al.*, 2019; Fong *et al.*, 2017).

AB-SA01 and AB-PA01, and their component phages were tested on 53 *S. aureus* and 41 *P. aeruginosa* isolates, respectively. The findings in the present study show strong infectivity and broad host range characteristics of all phage preparations. These findings are consistent to earlier studies findings that used similar test protocols, phages used in this study, and different phages (Lehman *et al.*, 2019; Zhang *et al.*, 2018; Fong *et al.*, 2017; Leskinen *et al.*, 2017; Hall *et al.*, 2012; Mendes *et al.*, 2014; Fu *et al.*, 2010; O'Flynn *et al.*, 2004).

Chapter 2 examined the lytic effect and host range of the phage cocktail AB-SA01 and its components. AB-SA01 is a mixture of J-Sa 36, Sa 83, and Sa 87 phages. AB-SA01 lysed about 89% of the isolates tested regardless of the antibiotic susceptibility profile of the isolates. The host range of phage cocktails could potentially be improved by incorporating other phage types, although it has been suggested that too many phage types in the therapeutic mixture could bring a negative impact on non-target bacteria (Alves *et al.*, 2014; Chan *et al.*, 2013). It was observed that AB-SA01 produced a strong lytic effect, exhibited by complete and clear lysis, on over 95% of the susceptible isolates than any of the component phages alone. This finding shows that resistance strain to one component phage could be treated by the other in the mixture as reported earlier (Lehman *et al.*, 2019; Mendes *et al.*, 2014).

Studies suggest the use of phage cocktails over single phage minimizes the emergence of resistant mutants (Gu *et al.*, 2012; Tanji *et al.*, 2005). In the present study, it was also found that at least 75.5% of *S. aureus* clinical isolates, including the MDR and MRSA, were sensitive to each phage-type of the phage cocktail. This finding shows that AB-SA01 and its components were effective irrespective of antibiotic susceptibility of the isolates. These findings are in line with previous studies conducted on the efficacy of phage cocktails and component phages of *S. aureus* (Leskinen *et al.*, 2017; Mendes *et al.*, 2014; Kelly *et al.*, 2011) and other bacterial species (Manohar *et al.*, 2019; Gundogdu *et al.*, 2016; Ozkan *et al.*, 2016; Gu *et al.*, 2012; Hooton *et al.*, 2011; Fu *et al.*, 2010; O'Flynn *et al.*, 2004).

The broad host range and strong lytic efficacy of phage cocktail AB-SA01 and its components led to the evaluation of the phage cocktail on biofilms in this study. During the evaluation, significant biofilm biomass reduction was observed on more than 79% of the isolates due to the lytic efficacy of AB-SA01. Not surprisingly, most resistant and intermediately susceptible *S. aureus* isolates to AB-SA01 during spot tests were also resistant in their biofilm form. This resistance was not unexpected because bacteria in the biofilm state develop more resistance to phages than in a planktonic state (Fu *et al.*, 2010). The resistance might be because bacterial cells in biofilms are metabolically less active and are less accessible for phage infection than bacterial cells in planktonic forms (Gutierrez *et al.*, 2015; Sutherland *et al.*, 2004). The effect of AB-SA01 on *S. aureus* isolates biofilm biomass reduction is consistent with earlier reports (Alves et al., 2014, Kelly et al., 2011). In chapter 2, it was shown that the use of phage cocktail AB-SA01

could provide an alternative to antibiotics in combating antibiotic-resistance, including MRSA and MDR *S. aureus,* infection in planktonic and biofilm states.

In chapter 3 of this thesis, the lytic efficacy of phage cocktail AB-PA01 and its component phages on *P. aeruginosa* laboratory strains and clinical isolates was examined. The study was conducted using spot tests and biofilms. The results show that each component phage and their mixture displayed strong lytic efficacy. Moreover, AB-PA01 demonstrated stronger lytic efficacy and a broader host range compared with each phage type during spot test. AB-PA01 produced a lytic effect on more than 93% of isolates, while the host range of the component phages varied within 65% – 88% of the isolates. It is expected this effect to increase with high MOI because high MOI reduces biofilms and prevent biofilm regrowth (Alves *et al.*, 2016; Abedon, 2009; Kasman *et al.*, 2002). Similar findings were reported previously (Leskinen *et al.*, 2017; Mendes *et al.*, 2014; Chan *et al.*, 2013; Hall *et al.*, 2012). AB-PA01 reduced biofilm biomass from more than 82% of *P. aeruginosa* isolates biofilms within a 12-hour treatment period. This biofilm biomass reduction indicates the lytic efficacy of AB-PA01 against its targets in planktonic and biofilm phases, irrespective of the antibiotic susceptibility profile of the isolates. Studies on various phage cocktails have also found similar results (Alves *et al.*, 2016; Gutierrez *et al.*, 2015; Fu *et al.*, 2010).

In chapters 2 and 3, it was observed that both AB-SA01 and AB-PA01 phage cocktails significantly reduce the biofilm biomass produced by their hosts in single-species biofilms. These results were obtained using a static biofilm model system on a polystyrene surface for 48

hours and applying phage cocktail treatment on the preformed biofilms for 12 hours. The effect of the phage cocktails was measured using crystal violet staining. Because the probability of occurrence of mutation is in the order of 1 x 10^{-8} strains (Matic *et al.*, 1997), a bacterium may need to undergo multiple mutations to achieve resistance.

The probability of resistance to the phage cocktail is lower than to single phage. If resistance occurs during phage cocktail therapy, it can be improved by including additional phage types to the therapeutic mixture. In the case of an *in vivo* situation, reducing the bacterial load could be enough to bring the bacterial density down to a level that can be controlled by the immune system or antibiotic treatment (Kirby *et al.*, 2014). Several studies have demonstrated that the use of a phage cocktail brings not only broad host range coverage but also prevents the development of phage resistant clones and failure of phage treatment (Alves *et al.*, 2016; Alves *et al.*, 2014; Gu *et al.*, 2012). Limited studies conducted on the effect of phage cocktails of *S. aureus* (Drilling *et al.*, 2014; Kelly *et al.*, 2012; Cerca *et al.*, 2007) and *P. aeruginosa* (Fong *et al.*, 2017; Pires *et al.*, 2011) showed a substantial reduction or elimination of biofilms produced by those species compared to PBS treated controls.

Following the treatment effect of phage cocktails, AB-SA01 and AB-PA01, on single species plate cultures and biofilms, the effect of these phage cocktails in mixed-species planktonic cultures and biofilm forms was investigated using fluorescently labelled laboratory strains and selected clinical isolates using bacterial cell count as a measurement of efficacy as discussed in chapter 4. Moreover, in chapter 4, the possibility of monitoring the effect of various phage

cocktails treatment effect and appearance of resistant mutants in real-time monitoring using red fluorescent protein labelled *S. aureus,* and green fluorescent protein labelled *P. aeruginosa* is demonstrated. Loss or significant decrease of mCherry or GFP fluorescence from the mixed-species bacterial populations of planktonic cultures was considered an indicator of the death of bacterial cells labelled with these fluorescence proteins. It is reported earlier that the decrease in fluorescence is an early and sensitive indicator of cell death (Steff *et al.*, 2001; Webb *et al.*, 2001). The decrease in fluorescence was correlated with a decrease in bacterial cell counts. Phage cocktails were as effective as gentamicin in killing bacteria in planktonic form, regardless of the antibiotic sensitivity profile of the bacterial hosts.

A high fluorescence signal in terms of relative fluorescence unit was observed in non-treated controls or bacterial species unaffected by the treatment. A decrease or increase in fluorescence intensity was associated with a decrease or increase in CFU, respectively. This observation was supported by reports that show the magnitude of fluorescence or absorbance, and colony count results are supplementary in showing bacterial density (Kong *et al.*, 2016; Russo *et al.*, 2015). The findings of experiments in chapter 4 show the feasibility of real-time monitoring of the efficacy of phage treatment using fluorescently labelled bacteria in mixed-species planktonic cultures. This assay combines rapidity and ease of management. Moreover, it is amenable to high-throughput screening setups for phage cocktail efficacy or antimicrobial agent evaluation.

Phage cocktail application on clinical isolates in mixed-species biofilms also resulted in bacterial cell reduction, suggesting that phage cocktails can control host cells in mixed-species biofilms.

The effect of phage cocktails in mixed-species biofilms followed similar patterns to those observed in single-species biofilm infections. The efficacy of phage cocktails in this study is consistent with the view that microheterogeneity of biofilms, with variable distribution of cells and presence of non-susceptible bacteria, does not prevent phages from reaching their hosts (Gutierrez *et al.*, 2015; Briandet *et al.*, 2008). This experiment demonstrates that phage cocktails can effectively kill target hosts within a mixed-species biofilm, in line with previous findings (Gonzalez *et al.*, 2017; Gutierrez *et al.*, 2015; Liao *et al.*, 2012; Sillankorva *et al.*, 2010; Briandet *et al.*, 2008; Harcombe and Bull, 2005).

In this study, it was seen that phages work more quickly on planktonic cultures than on biofilms, perhaps because bacterial cells in biofilms are in a lower metabolic activity state; hence phages cannot proliferate as efficiently and quickly compared to in free and actively growing cells in a planktonic state (Cerca *et al.*, 2007). Phage cocktails AB-SA01 and AB-PA01 are shown to be capable of killing their hosts in planktonic and biofilm phases in mixed-species cultures, as demonstrated in chapter 4. The findings in this chapter demonstrate that phage cocktails, alone or in combination, can kill their hosts in the presence of non-susceptible hosts in planktonic and biofilm phases. The data presented in this chapter strongly support the potential of the lytic phage cocktails in the treatment of antibiotic-resistant hosts in mixed-species infection both in planktonic and biofilm phases.

It is essential to corroborate the *in vitro* efficacy of phages using appropriate animal infection models. Studies indicated that *in vitro* efficacy of phages may not always correlate with the *in vivo* efficacy because the *in vivo* situation is more complicated as the physiological state of the

bacteria, including phage receptors expression, could be different (Tsonos *et al.*, 2014; Henry *et al.*, 2013). A mouse excisional wound model modified using silicone sheet splint to mimic the re-epithelization and granulation type of wound healing in humans is advised to evaluate the outcome of new treatment agents and wound healing *in vivo* (Davidson *et al.*, 2013; Dunn *et al.*, 2013).

Although rendering mice diabetic is challenging, the injection of STZ is reported to be effective and rapid in inducing type 2 diabetes mellitus (Deeds *et al.*, 2011). Following the established procedure, IP STZ injection was used as detailed in chapter 5 of this thesis. This experiment was successful in terms of producing a high proportion of diabetic mice, similar to the results of recent studies (Deeds *et al.*, 2011; Hayashi *et al.*, 2006). The excisional splint wound model employed in the present study facilitated re-epithelization and granulation wound healing similar to that in humans. Diabetic mice skin excisional wounds were infected with MDR *S. aureus* and treated with phage cocktail AB-SA01 topically in multiple doses at high phage titer (9.0 log_{10} (PFU/ml)). Phage treatment studies on chicken skin (Goode *et al.*, 2003) and Balb/c mice burn wounds (Kumari *et al.*, 2010) show that low-titter phage treatment is unlikely to be successful. The mice tolerated phage cocktail AB-SA01 used in this study well since no adverse event or clinically manifested abnormality was observed in uninfected phage-treated wounds. In infected and treated wounds, adverse effects due to rapid bacterial lysis (Capparelli *et al.*, 2007; Dixon, 2004) were not observed as well.

Chapter 5 showed bacterial load reduction to the complete arrest of infection due to phage cocktail AB-SA01 treatment, and AB-SA01 efficacy was at least similar to vancomycin. Most of the phage cocktail treated wounds did not produce a detectable amount of *S. aureus* at the end of the experiment. The result of this multiple-dose scheme was similar to studies on *Salmonella* phages (Bardina *et al.*, 2012) and *E. coli* phages (Huff *et al.*, 2003) efficacy using chicken infection models that reported better results of repeated doses than the single dose. As opposed, a study that used only one phage on *ex vivo* human skin indicated that multiple doses do not improve the treatment outcome compared with a single dose treatment (Vieira *et al.*, 2012). In terms of wound healing, the topical administration of AB-SA01 produced a similar result to vancomycin IP treatment in hastening wound healing. This finding agrees with previous reports (Chhibber *et al.*, 2013; Mendes *et al.*, 2013). Most of the wounds from infected phage- and vancomycin-treated mice were completely closed, and hair regrowth was observed on some of the closed wounds. The wound size measurement scores also strongly associated with the decreased bacterial load.

Appropriate debridement is the standard procedure in wound management, especially before the application of topical antimicrobial (Nusbaum *et al.*, 2012), and phage treatment is not the exception (Mendes *et al.*, 2012a). Reports show that phage therapy will be better effective if applied after debridement (Jault *et al.*, 2019; Seth *et al.*, 2013; Mendes *et al.*, 2012a). In this study, the efficacy of topical phage cocktail treatment with gentle debridement was evaluated. The results on the efficacy of topical phage cocktail in diabetic mice wound infection in terms of bacterial load reduction and wound closure obtained in the current study represent the basis for

the initiation of human clinical trials to explore the potential of topical phage cocktail in the treatment of DFU and other chronic skin wound infections. The present wound infection treatment model: debridement, topical application of the phage cocktail, and coverage with a light dressing such as gauze and Opsite, may represent an appropriate blueprint for better chronic wound care.

6.2 Directions for future research

Wound healing outcomes depend on load and virulence of the infecting bacterial species, presence of antibiotic resistance factors including biofilms, immune status of the patient, and the efficacy of therapeutic agents. This study examined the lytic efficacy of phages on *in vitro* planktonic and biofilm phases and animal models using bacterial cell and biofilm biomass reduction as efficacy measurements. There are limited reports that phage-resistant mutants are less virulent (Hall *et al.*, 2012; Kutter, 2009; Capparelli *et al.*, 2007). A detailed investigation is needed to examine the phage resistance character and pathogenicity of surviving bacteria.

The present study did not evaluate the lytic effect of phage cocktails in multiple bacterial species infected wounds. This assessment is essential because most of the DFU infections are caused by more than one bacterial species (Mottola *et al.*, 2016; Ramakant *et al.*, 2011). The effect of phage therapy on healing of wounds infected by multiple bacterial species cannot be easily projected because of complex interactions among phages, infecting microbial species, and infected patient immune system.

This study also did not assess the effect of phage treatment on immune modulation and microflora of the skin of mice in the treatment area. A report on the effect of topical application of phages indicated that phage neutralization might not be a significant problem as the kinetics of phages are much rapid than antibodies production (Sulakvelidze *et al.*, 2001). Hence, research should be conducted to clarify these doubts. Examination of swab and skin samples collected throughout an experiment will be necessary for the proper assessment of the effect of phage

therapy on wound microbiome and examine the next dominant bacteria after the phage therapy. This microbiome assessment before and after phage treatment will help to examine the off-target effects of phages. Studies that assess the benefit of multispecies phages cocktails to reduce or eliminate multispecies biofilms should also be conducted.

Recent studies suggest that phages can evade bacterial host adaptive immunity, mainly CRISPR-Cas and restriction-modification systems, through protecting their genome using different proteins (Mendoza *et al.*, 2020; Malone *et al.*, 2020). These specific protein-based defence strategies should be investigated thoroughly. Moreover, a study indicated that phages could modulate human or animal immune responses through inducing anti- and pro-inflammatory responses (Van Belleghem *et al.*, 2017). A further in-depth study is recommended to examine the interaction of phages with the immune system and other possible side effects.

Studies on the cytotoxicity of phages are limited, particularly in wound infection models (Rose *et al.*, 2014; Mendes *et al.*, 2013). The findings of mice wound infection models in the present study show that phage therapy resulted in wound healing and significant bacterial load reduction. However, this study did not specifically evaluate the quality of wound healing. The clinical application of topical phage cocktail therapy may be more appropriate in the context of severe DFU infections that involve bones. So, future investigations of this practice on animal models of osteomyelitis are recommended. Formulations of phages for clinical application, including ointments or droplets, phage-impregnated wound dressings, and nanoparticle-based phage preparations, should be studied and developed.

In studies that involve laboratory animal infection models, the dose of phage cocktails needed to eliminate or reduce the infection to the level that can be controlled by the immune system or antibiotic treatment must be determined. The dose determination study should be conducted considering the “active” phage therapy principle that relies on self-amplifying and self-limiting character of phages to meet the necessary dose (Loc-Carrillo and Abedon, 2011).

In this study, the efficacy of a phage cocktail alone was evaluated against MDR *S. aureus* on the infected mice wound model. Studies coupling the use of phage cocktails with antibiotics on several clinical isolates should be future research focus. Collaboration research is required to take the findings of this study further to toxicological and clinical trials level.

The main goal of most of *in vitro* and pre-clinical experiments in antimicrobial therapy is to evaluate the efficacy of antimicrobial agents to pave the way to clinical trials. In line with this, the results of this study are important and need to be translated into further preclinical and clinical trials. Hence, the next phase of this study that will start within a few months to evaluate the application of the phage treatment using phage fixed on the wound dressing and gel form on animal models. Based on the results of the animal experiments, the next level study including clinical trial phase I/II will be recommended.

The treatment failure due to increasing resistance of bacterial infections to antibiotics forced scientists to search natural alternatives for treatment including phages. Phage libraries that identify and preserve mono- or cocktail-based phage therapy using the full spectrum of bacterial strains available in the clinical microbiology laboratories of a specific region or country is

needed. Hence, the shelf-ready phage strains or cocktails can reasonably cover antibiotic-resistant bacteria treatment in that region or country. Modern phage therapy has been suffering from insufficient credibility, patient and physician unfamiliarity and limited product availability. Currently, there is a limited supply of phages and phage cocktails as few phage companies or research institutions are working on phages. The results of this study are a good encouragement for phage industries. Therefore, collaborative efforts should be made among industries, governmental institutions, and charities to establish phage libraries and encourage phage commercialization. Governments and the World Health Organization should formulate appropriate regulations and intellectual property right that consider the unique characteristics of phages.

7. Appendices

The following publications and presentations have been produced from the chapters of this thesis.

Publications

Legesse Geredew Kifelew, James G. Mitchell & Peter Speck. Mini review: efficacy of lytic bacteriophages on multispecies biofilms. *Biofouling*, 2019, 1-10.

Legesse Garedew Kifelew, Morgyn S. Warner, Sandra Morales, Nicky Thomas, David L. Gordon, James G. Mitchell and Peter G. Speck. Efficacy of Lytic Phage Cocktails on *Staphylococcus aureus* and *Pseudomonas aeruginosa* in Mixed-Species Planktonic Cultures and Biofilms. *Viruses*, 2020, 12 (559), 1 - 16.

Legesse Garedew Kifelew, Morgyn S. Warner, Sandra Morales, Lewis Vaughan, Richard Woodman, Robert Fitridge, James G. Mitchel, and Peter Speck. Efficacy of Phage Cocktail AB-SA01 Therapy in Diabetic Mouse Wound Infections Caused by Multidrug-Resistant *Staphylococcus aureus*. BMC Microbiology (in press), 2020, 1 - 16.

Presentations

Legesse G. Kifelew, Morgyn Warner, Sandra Morales, Lewis Vaughan, James G. Mitchell, and Peter Speck. Efficacy of Phage AB-SA01 Therapy in Diabetic Mice Skin Wound Infections Caused by Multidrug Resistant *Staphylococcus aureus*. *Australasian Virology Society Conference*, Queenstown, New Zealand, 2 - 5 December 2019.

Legesse G. Kifelew, Morgyn Warner, Sandra Morales, Lewis Vaughan, James G. Mitchell, and Peter Speck. Efficacy of Phage AB-SA01 Therapy in Diabetic Mice Skin Wound Infections Caused by Multidrug Resistant *Staphylococcus aureus*. *Evergreen Phage Meeting,* Olympia,

WA, USA, 4-9 August 2019, and *Diabetic Foot Australia Conference*, Brisbane, Queensland, AU, 8-10 September 2019.

Legesse G. Kifelew, Morgyn Warner, Sandra Morales, Nicky Thomas, David Gordon, Robert Fitridge, Peter Speck (2018). Bacteriophage efficacy against *Staphylococcus aureus* and *Pseudomonas aeruginosa ex vivo* isolates from diabetic foot ulcer (DFU) patients. *Oxford Bacteriophage Conference – Phages 2018*, Oxford, UK, 11-12 September 2018.

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Mini-review: efficacy of lytic bacteriophages on multispecies biofilms

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ABSTRACT

There is potential for phages to prevent and control bacterial biofilms, but few studies have examined the effect of phages on the multispecies biofilms that characterize most bacterial infections. This paper reviews the mechanism of action of phages, the evidence supporting the view that phage therapy will be effective against bacterial targets and the opposite viewpoint, phage application approaches, and the comparative advantage of phage therapy in multispecies biofilms. The few reports measuring the actions of lytic phages against multispecies biofilms are also reviewed. The authors are cautiously optimistic about the application of phages against their targets when in multispecies biofilms because some lysis mechanisms do not require species specificity.

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Bacteria; biofilm; efficacy; multispecies; phage; susceptible

Introduction

Human lifespan and quality of life are being compromised by the substantial rise in antimicrobial-resistant bacterial pathogens (Smith et al. 2015; Speck 2013). Unlike previous decades, there are few antimicrobials in development to counter the resistant microbes (WHO, 2014), perhaps in part because large pharmaceutical companies are ceasing to invest in this area (Projan 2003), despite microorganisms continuing to evolve and major challenges such as implant biofilms being unresolved (Kaur et al. 2016; Yilmaz et al. 2013). These microorganisms share a common milieu and coexist by forming surface-associated multispecies biofilms (Woods et al. 2012; Thein et al. 2007). Two-thirds to three-quarters of human infections are thought to be biofilm-related (Seth et al. 2013). Moreover, most of these infections involve multispecies biofilms (Peters et al. 2012). Multispecies biofilms in a single community can provide benefits to the species involved, in the form of genetic material exchange, metabolic cooperation, quorum sensing system sharing, development of passive resistance, niche optimization, host immune modulation, virulence induction, and by-product influences that give the involved species competitive advantages (Shettigar et al. 2016; Tay et al. 2016; Wolcott et al. 2013). Bacterial biofilms are more resistant to biocides than planktonic cells due to the extracellular matrix that comprises most of the biofilm, and the bacterial cells in the biofilms that become metabolically inactive (Harper et al. 2014). This may complicate the infection and treatment outcome (Pastar et al. 2013; Dalton et al. 2011).

Bacteriophages ("phages", viruses that infect and may kill bacteria) are proposed as a means of ameliorating the antibiotic resistance crisis (Bhattacharjee et al. 2015; Yilmaz et al. 2013, Seth et al. 2013). Although the use of phages as antibacterial agents was started 100 years ago, humankind has not benefitted significantly from phage therapy to date. This is partly because of the introduction of antibiotics a half a century ago (Wei 2015; Golkar et al. 2014), scientific uncertainties about the co-evolutionary aspect of the phage–bacterium couplet (Pirnay et al. 2015; Høyland-Kroghsbo et al. 2013; Maura and Debarbieux 2012), poor understanding of what phages were or how they functioned (Storms and Sauvageau 2015; Pirnay et al. 2011; Kutter et al. 2010; Young 1992), scepticism toward efficacy of phage therapy (Young and Gill 2015; Henry et al. 2013; Krüger and Bickle 1983), absence of compatible regulatory frameworks for phage therapy (Verbeken et al. 2012), the difficulty of patenting phages (Kutter et al. 2015; Pirnay et al. 2012), and the lack of standardized criteria for phage preparation, purity, and potency (Merabishvili et al. 2009; Housby and Mann 2009; McVay et al. 2007; Marza et al. 2006; Wills et al. 2005).

High efficacy, bacterial specificity, quick onset of action, and low risk of resistance, if used in multi-

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phage cocktails, now make phage therapy a feasible option for use in humans (Pastagia et al. 2011). While lytic phages appear to be efficacious against bacteria within single-species biofilms, little is known about the lytic efficacy of phages when their target is contained within multispecies biofilms (Alves et al. 2016; Drilling et al. 2014; Carson et al. 2010). This paper will outline pertinent biofilm and phage characteristics, and the evidence for when phages will or will not maintain lytic efficacy in multispecies biofilms.

Bacterial biofilms

A bacterial biofilm is a group of bacteria attached to each other and usually to a surface (Allewell 2016; O'Toole et al. 2000; Skillman et al. 1998). The bacterial community in the biofilm is encased in an extracellular matrix; bacteria in this form are found ubiquitously in the environment (Teh et al. 2014; Kostakioti et al. 2013). This matrix is mainly composed of polysaccharides, proteins, lipids and DNA, and is often referred to as extracellular polymeric substance (EPS) (Teh et al. 2014; Karunakaran et al. 2011). Studies of EPS show that it reduces microbial mobility and limits the transfer of nutrients and oxygen (Steenackers et al. 2016). In addition, the matrix contributes to the development of an anaerobic environment and structural heterogeneity in biofilms (Lacroix-Gueu et al. 2005). Inside the matrix, the biofilm may consist of densely populated single or multiple species of bacteria (De Beer and Stoodley 2006). Scanning electron microscopy and related techniques reveal the micro-heterogeneity of biofilms, with variable distribution of cells, matrix, and fluid-filled channels and pores (Wood et al. 2000). Natural biofilms are a mixture of micro-organisms sharing a common milieu and coexisting in niches by forming multispecies biofilms (Thein et al. 2007). Natural biofilms are usually polymicrobial, and bacteria occupy 5%–30% of the volume of the biofilm (Zhao et al. 2013).

Of all human infections, 65–80% are biofilm-related, and biofilm formation is often considered the underlying reason for antibiotic treatment failure (Coenye and Nelis 2010). Bacteria residing in biofilms are more tolerant to physical and chemical disruptions than their planktonic counterparts (Steenackers et al. 2016; Gilbert et al. 2002). Studies have also shown that bacteria in biofilms are 100-1000 times more resistant to antibiotics than in the planktonic phase (Burmolle et al. 2006; Ceri et al. 1999). Bacterial resistance to antibiotics in biofilms is mainly due to the complex nature of the EPS that give mechanical stability to the biofilm and serves as an external digestive system that keeps extracellular enzymes near the bacteria (Flemming and Wingender 2010). The extracellular matrix also protects bacteria since it reduces the access of solutes to the bacteria through the combination of ionic interaction and molecular sieving (Allison 2003). The presence of metabolically inactive persister cells also contributes to the antibiotic resistance of bacteria in biofilms (Harper et al. 2014).

Phages and their mechanism of action in biofilms

The mechanism by which phage spreads through biofilms and how they kill their hosts appears to be that lytic phages encode or stimulate their hosts to produce EPS-degrading enzymes, which facilitate their movement through biofilms (Sutherland et al. 2004). Then, phages move through biofilms, proliferate within their host bacteria, and finally eliminate their hosts *via* lytic activity (Zhang et al. 2014; Gilbert et al. 2002) as illustrated in Figure 1. Some phages carry genes to encode production of enzymes by the host bacteria that degrade bacterial capsules and other EPS; for example *Bacillus subtilis* phage ΦNIT1 encodes a monomeric 25-kDa degradation enzyme (designated γ-PGA hydrolase, PghP) to break down poly-γ-glutamate (γ-PGA) - a host capsular polypeptide of glutamate with a γ-linkage, so that phage progenies easily infect encapsulated cells (Kimura and Itoh 2003). However, phage adsorption and proliferation does depend on the growth phase of the bacterial host. Phage entry and multiplication will be enhanced when phages are applied during an exponential growth phase of the bacteria. But dormant and dead host cells will make it difficult for the phages to migrate through and multiply in the biofilm that may lead to abortive infection. This difficulty will be exacerbated if these dead and dormant host cells are present in large numbers (Hu et al. 2012).

In multispecies biofilms, various structures of biofilm including EPS and heterologous microbial cells may impede phage access to the host cell surface (Sutherland 1999; Sutherland and Wilkinson 1965). The physiological state of the host cells such as stationary and declining growth phases, strong co-aggregation with other bacteria, tight cell-cell binding, and unavailability of phage receptors on their surfaces will affect the outcome of phage attack (Rickard et al. 2003). Some phages possess an enzyme, produced in the bacterial host due to their encoding effect, in their

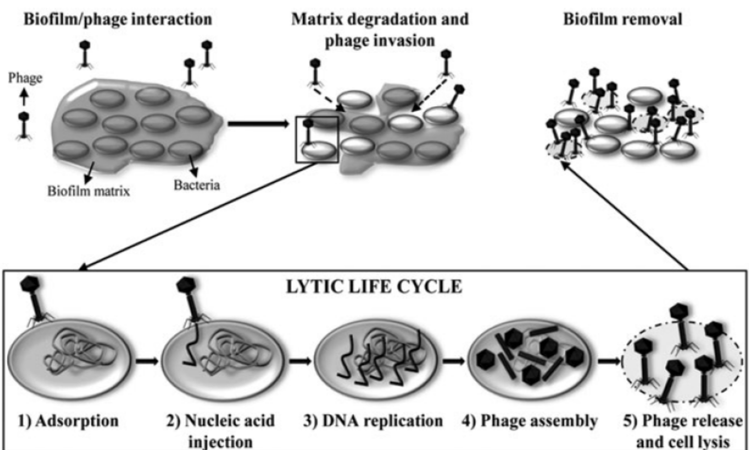

Figure 1. Mechanism by which phages disrupt biofilms (taken from Gutierrez et al. 2016).

spikes (Pelkonen et al. 1992; Kwiatkowski et al. 1982; Rieger-Hug and Stirm 1981; Saxelin et al. 1979; Bessler et al. 1975). Once the phage and biofilm make contact, the biofilm matrix is thought to be degraded by these phage-associated hydrolytic enzymes termed polysaccharide depolymerases (Yan et al. 2014; Carson et al. 2010; Kimura and Itoh 2003). Most double-stranded phages encode hydrolytic enzymes and lyse the host bacteria at the last step of the lytic infection cycle (Gutierrez et al. 2016; Hughes et al. 1998). Phages are released as they achieve local lysis of susceptible cells and as associated enzymes degrade EPS within the biofilm. Some phage-associated depolymerases are highly specific for host-derived EPS and may have limited effects on multispecies biofilms (Chan and Abedon 2015; Nelson et al. 2001). There are also some capable of degrading the EPS of many genera (Deveau et al. 2002; Sutherland et al. 2004; Yoong et al. 2004; Pei and Lamas-Samanamud 2014).

Phages with depolymerase enzyme can readily reach the host cell surface by digesting their way as described by earlier studies (Gutierrez et al. 2016; Yan et al. 2014; Sutherland et al. 2004; Hughes et al. 1998). Phages penetrate the bacterial cell wall using their enzyme or by contracting a sheath and injecting their genome into the cytoplasm of the host bacterium (Figure 1, step 2). Inside the bacterium, the phage replicates its genome and synthesizes its enzymes and structural components using the host replication and metabolic pieces of machinery, respectively. Phage structures synthesis and replication can be achieved through the expression of phage early genes to regulate these machineries (Figure 1, step 3). By the expression of the phage late genes, assembly of the phage structures, packaging of the genome, and maturation will be carried out to produce phage particles (Figure 1, step 4) (Gutierrez et al. 2016). Finally, phage coded lysozyme breaks down the peptidoglycan layer of the host bacterium causing lysis and release of the new phage progeny ready to infect other similar bacterial cells (Figure 1, step 5) and remove the biofilm through the action of polysaccharide depolymerases (Gutierrez et al. 2016; Harper et al. 2014; Hughes et al. 1998).

Evidence of lytic efficacy of phages in multispecies biofilms

Although many studies have examined the efficacy of phages on planktonic bacteria and single-species biofilms, few have examined the efficacy of phages against multispecies biofilms. One such study (Kay et al. 2011) assessed the effects of λW60 (*Escherichia coli* phage) and PB-1 (*Pseudomonas aeruginosa* phage) on mono- and dual-species planktonic and biofilm cultures of *E. coli* and *P. aeruginosa*. This study found that phages achieved a higher titre and depleted their bacterial host population more effectively within multispecies biofilms compared to planktonic or single species biofilm treatment. This could be evidence that shows the efficacy of phage against bacteria in the multispecies biofilms (Kay et al. 2011).

The importance of phage dependent depolymerase to degrade bacterial EPS has been known for over 60 years (Sutherland and Wilkinson 1965). Reports in the late 1990s indicated that the disruption of a biofilm by phages is a combination of EPS degradation by the depolymerases and infection and subsequent cell lysis by the phages (Hughes et al. 1998). Some phages can induce their host to produce and release

depolymerases (Yan et al. 2014; Hanlon et al. 2001; Hughes et al. 1998; Sutherland and Wilkinson 1965; Adams and Park 1956). Some phages can also contain depolymerases by their particles or spikes (Pei and Lamas-Samanamud 2014; Son et al. 2010; Sutherland et al. 2004; Pelkonen et al. 1992; Kwiatkowski et al. 1983). These reports showed that depolymerase-loaded or -inducing phages can more strongly disrupt multispecies biofilms through enzymatic degradation of bacterial EPS than non-depolymerase-possessing or non-depolymerase-inducing phages. This appears to be because enzymes released from the surface of the phages or the bacterial host cells during lysis can rapidly break down biofilms (Pei and Lamas-Samanamud 2014). Thus, phages that contain or induce depolymerase production by their host could access the host bacteria more readily in the biofilms than phages without depolymerases (Harper et al. 2014).

A study finding on a phage cocktail comprising ØIBB-PF7A (a phage against *Pseudomonas fluorescens*) and ØIBB-SL58B (a phage against *Staphylococcus lentus*) on mono- and dual-species biofilms indicated that the phage cocktail effectively removed both bacterial hosts from all strata of the biofilm (Sillankorva et al. 2010). The finding also showed that ØIBB-SL58B and ØIBB-PF7A were able to multiply in the dual-species biofilms; ØIBB-PF7A reached its host when the host population was present in the minority and the majority in the dual-species biofilms. Moreover, the finding demonstrated that the phage cocktail caused a significant decrease in the number of bacteria released from the biofilms into the planktonic phase, and phage **Ø**IBB-PF7A produced significant damage to the dual-species biofilm. It also indicated that the damage to the biofilm due to **Ø**IBB-PF7A facilitated the release of non-susceptible *S. lentus* to the environment (Sillankorva et al. 2010). Non-susceptible bacteria and their extracellular matrix do not prevent phages from reaching their hosts (Briandet et al. 2008). A similar study that evaluated the efficacy of 5 phages on dual-species biofilms of *Enterobacter cloacae* NCTC 5920 and *Enterobacter agglomerans* also demonstrated that application of a cocktail of phages against each bacterial species could eliminate both bacterial hosts from dual-species biofilms (Tait et al. 2002).

In an investigation using two-photon excitation *in situ* fluorescence correlation spectroscopy, the activity of lactococcal c2 phage against biofilms formed by c2-non-susceptible *Stenotrophomonas maltophilia* strain 114N-Sm, c2-susceptible *Lactococcus lactis* subsp. *cremoris* OSM31, and c2-resistant *Lactococcus lactis* subsp. *lactis* IL-1403 was examined. The lactococcal c2 phages penetrated and diffused through multispecies biofilms without being affected by EPS or non-susceptible hosts. In addition, the authors explained that the microheterogeneity of biofilms with a variable distribution of cells, EPS, and water-filled channels did not affect phage access into the interior of the biofilms (Briandet et al. 2008). A similar study conducted using *P. aeruginosa* phage ΦE2005-A on dual-species biofilm formed by susceptible *P. aeruginosa* and non-susceptible *E. coli* found that application of the phage ΦE2005-A on *P. aeruginosa-E. coli* dual-species biofilms effectively removed *P. aeruginosa* in the presence of non-susceptible *E. coli* species (Liao et al. 2012).

The lytic efficacy of *S. lentus* phage fIBB-SL58B and *P. fluorescens* phage fIBB-PF7A against their hosts in the dual-species biofilm that consists of *P. fluorescens* and *S. lentus* also support the research findings that indicated phages can effectively remove their hosts in the biofilms formed by their hosts and other bacteria (Sillankorva et al. 2010). Evaluation of *E. coli*-specific phages (T7, T5 or both) for their effect against *E. coli* on *E. coli-Salmonella* Typhimurium dual-species biofilms showed that these phages successfully reduced the *E. coli* population. The evaluation result also indicated that application of *S.* Typhimurium phage SP6 on *E. coli-S.* Typhimurium dual-species biofilms led to a decreased *S.* Typhimurium population in the presence or absence of *E. coli.* Consequently, it concluded that the capability of phages to reach their hosts could not be compromised by the presence or absence of non-susceptible bacteria in multispecies biofilm (Harcombe and Bull 2005). Gutiérrez et al. (2015) also reported at least a 2-log unit reduction in the population of adhered *Staphylococcus aureus* by phiIPLA-RODI phage and *Staphylococcus epidermidis* by phiIPLA-C1C phage from *S. aureus-S. epidermis* dual-species biofilms. The authors suggested that this finding complemented previous research that documented the efficacy of phages in removing their hosts from multispecies biofilms and concluded that lytic phages are effective biofilm disrupting agents, including for multispecies biofilms in the presence of non-susceptible hosts (Gutierrez et al. 2015).

The efficacy of *S. aureus* phage phiIPLA-RODI on biofilms formed by *S. aureus* in combination with *Lactobacillus plantarum*, *Enterococcus faecium*, or *Lactobacillus pentosus* was reported and the level of effectiveness of phage treatment on multispecies biofilms varies depending on the bacterial species accompanying the host bacteria (González et al. 2016). The

propagation of phage phiIPLA-RODI was higher in biofilms formed by *S. aureus-L. pentosus* than in biofilms formed by *S. aureus* with the remaining two bacterial species. Scanning electron and confocal laser scanning microscopy also showed that phiIPLA-RODI treatment made the biofilms flatter and less organized and decreased intact *S. aureus* cells compared with untreated samples.

The search for effective use of phages in the treatment of biofilms continues to advance. Use of quorum-quenching phages that can lyse host bacteria and express quorum-quenching enzymes to affect diverse bacteria in multispecies biofilms could be one potential area to make the best out of phage therapy (Pei and Lamas-Samanamud 2014). Pei and Lamas-Samanamud (2014) reported the lytic efficacy of engineered phage T7 which was encoded with the acyl homoserine lactones (AHL) lactonase AiiA gene to produce lactonase. They further detailed in the report that phages encoding lactonase have the capacity of quenching quorum sensing through degrading AHLs that are necessary for biofilm formation by facilitating bacterial cells communication. The report was the result of a study performed on a mixed biofilm of *E. coli* and *P. aeruginosa* after proving the existence of synergism in biofilm formation between the two species and knowing that *E. coli* (host for the phage) cannot synthesize AHLs but can respond to AHL released by other bacteria and *P. aeruginosa* can synthesize and respond to AHLs. According to the report, the addition of lactonase producing phage T7 to multispecies biofilms containing *P. aeruginosa* and *E. coli* can inhibit biofilm formation and disrupt biofilms by affecting both bacterial species. It further suggested that phages that lyse host bacteria and express quorum-quenching enzymes to affect diverse bacteria in multispecies biofilm communities will be a potential alternative treatment against multispecies biofilm infections in the future.

Polyvalent phages (broad-host-range phages) represent a potentially effective strategy against multispecies biofilms (Kim et al. 2012; Lin et al. 2012; Sillankorva et al. 2010; O'Flaherty et al. 2005; Jensen et al. 1998). For instance, the polyvalent PA1Ø phage is an efficient lysing agent for mixed-biofilm of *P. aeruginosa*, *S. aureus*, *S. epidermidis*, and *Staphylococcus hominis*. Consequently, PA1Ø is a useful alternative antimicrobial agent in the treatment of mixed biofilm-related infections, particularly of *P. aeruginosa* and *S. aureus* (Kim et al. 2012). These reports conclude that phages can control their hosts in most circumstances (Sillankorva et al. 2010). The capacity of phages to infect multiple bacterial host species can maximize opportunities for effective phage proliferation in multispecies biofilms since phages that are capable of interspecies or intergeneric infection will have increased chances to encounter suitable prey and replicate (Kim et al. 2012; Jensen et al. 1998). The ability of lytic phages to degrade multispecies biofilms, replicate within their host, and lyse the host even when applied at lower doses is documented in recent studies (Gutierrez et al. 2015; Chhibber et al. 2015; Sillankorva et al. 2010).

Findings of reduced lytic efficacy of phages in multispecies biofilms

Studies showing the reduced lytic efficacy of phages in multispecies biofilms are fewer in numbers compared to the number of studies showing good lytic efficacy of phages in multispecies biofilms. One possible reason for the reduced efficacy of phages in multispecies biofilms could be the limitation of phage particle migration by the presence of the EPS (Hughes et al. 1998). The diversity of EPS and the heterogeneous distribution of biofilm-forming bacteria and their EPS throughout a biofilm would hinder the action of phage depolymerase in multispecies biofilms (Neu and Lawrence 1997). This may be because some of the phage depolymerases are highly specific to targetting EPS chemistry (Hanlon et al. 2001). An experiment conducted on the activity of PB-1 and λW60 phages on dual-species biofilm formed by *P. aeruginosa* strain PAO1 and *E. coli* strain MG1655 using transmission electron microscopy also showed that phages can be trapped in the EPS component of multispecies biofilms. The result of the experiment indicate that infectivity of PB-1 and λW60 in multispecies biofilm was similar to that seen in planktonic cells when applied with EPS dissolving 0.5% Tween-20 but weaker when these phages are applied on multispecies biofilm in the absence of 0.5% Tween-20 (Kay et al. 2011).

Multispecies biofilm formed by *Microbacterium phyllosphaerae, Shewanella japonica, Dokdonia donghaensis*, and *Acinetobacter lwoffii* showed an increase in biomass of >167% compared to single species biofilm biomass due to the synergetic interaction of participating bacterial species. Furthermore, multispecies biofilms formed by these bacteria displayed higher resistance to other invading bacteria and antibacterial proteins than biofilms formed by any of these bacterial species alone (Burmolle et al. 2006). The resistance observed in multispecies biofilms against bactericidal

proteins may hold true for phage activity against multispecies biofilms since the large biofilm biomass or the complex EPS may hinder phage penetration of the biofilm or entrap the phages (Kay et al. 2011) or it may deter the activity of phage depolymerase (Hughes et al. 1998). The complex EPS may reduce the efficacy of phages because of entrapment of phage particles in the biofilm matrix (Kay et al. 2011), reduce multiplication of phages due to the large proportion of metabolically inactive host cells (Harper et al. 2014), or shedding of phage receptors from the host bacteria (Teplitski and Ritchie 2009).

The reduced lytic efficacy of phages on multispecies biofilms was also reported by Tait et al. (2002) who evaluated the efficacy of 5 phages of *E. cloacae* NCTC 5920 and *E. agglomerans* on single- and dual-species biofilms. These authors reported that *E. cloacae* NCTC 5920 could be removed by only 1 of the 5 phages; whereas *E. agglomerans* could be removed by 4 of the 5 phages used during the treatment of the dual-species biofilms. Hence, not all phages remove a susceptible bacterial host from multispecies biofilms. Since the complete elimination of *E. cloacae* NCTC 5920 was not achieved during the study, structural heterogeneity of multispecies biofilms through producing pockets of unattainable phage-susceptible bacteria was suggested as a possible reason. Based on these findings, it possible to conclude that the presence of non-susceptible bacterial populations could protect phage-susceptible bacteria from phage attack through creating 'spatial refugees' restricted in space and protected, within the biofilm (Tait et al. 2002). The common phage resistance mechanisms of bacteria including preventing phage adsorption, blocking entry or cutting phage nucleic acids, and abortive infection systems, can be used by the bacteria persisting inside the multispecies biofilms (Labrie et al. 2010).

Approaches to improve phage efficacy against multispecies biofilms

While it is broadly accepted that phages typically target a single bacterial species, this view might be biased by the methods used to discover phages, which usually involve the appearance of plaques on lawns of purified cultures of bacteria. A report describing phages isolated from Lake Michigan, USA by Malki et al. (2015), with a host range spanning several phyla of bacteria, shows that individual phage species may have a much broader target range than had been considered possible. The use of phages with a broad host range that can attack various bacterial species in multispecies biofilm would help in targeting such biofilms. It is possible to optimize the benefit of phages in controlling multispecies biofilms through facilitating their penetration. Li et al. (2017) found that use of polyvalent phages conjugated with magnetic colloidal nanoparticle clusters, in the presence of a magnetic field, achieved an enhanced penetration into biofilms.

Phages could be engineered for the extension of their host range in many ways, including homologous recombination of phages with plasmids that can alter phage tail fibre proteins (Lin et al. 2012) and successive passage of phages through the less sensitive target hosts (O'Flaherty et al. 2005). Genetically engineered phages could potentially be developed with enhanced capabilities for extracellular matrix dispersion, biofilm dissolution, and obstruction of bacteria-bacteria cell communication (Pei and Lamas-Samanamud 2014; Lu and Collins 2007). Pei and Lamas-Samanamud (2014) engineered T4 phage to encode a lactonase (a metalloenzyme, produced by certain species of bacteria and which inactivates AHLs) with broad-range activity for quenching of quorum sensing. Their work showed that genetic modifications of phages could enable phages to synthesise quorum quenching enzymes for higher efficacy against multispecies biofilms (Pei and Lamas-Samanamud 2014).

Phage cocktails have also been found to exploit the synergistic effect of component phages which could facilitate their adsorption and diffusion in multispecies biofilms. For instance, depolymerase produced by one phage can hydrolyse the EPS structure of the multispecies biofilm contributed from its host and closely related species. This may enhance the ability of the other phage to reach its host (Chhibber et al. 2015; Sillankorva et al. 2010). More effective removal of *P. aeruginosa* and *Klebsiella pneumoniae* from the mixed-species biofilm of the two bacterial species was observed when treated with *K. pneumoniae*-specific phage KPO1K2 and *P. aeruginosa*-specific phage Pa29 cocktail than treated by the respective phages alone. This could be attributed to the depolymerase coded by phage KPO1K2 that hydrolyzed the top layer of *K. pneumoniae* and *P. aeruginosa* from the multispecies biofilm and facilitated the entry of the two phages to reach the bacterial cell part of the biofilm (Chhibber et al. 2015). Similarly, the exposure of dual-species biofilms formed by *P. fluorescens* and *S. lentus* to a phage cocktail containing *P. fluorescens* phage fIBB-PF7A and *S. lentus* phage SL58 effectively removed the biofilm and killed their hosts (Sillankorva et al. 2010).

Conclusions

The clearance of multispecies biofilms through the application of phages is feasible but remains a challenging proposition. The available options to ensure that phages maintain lytic efficacy against multispecies biofilms include the formulation of phage cocktails that include phages against multiple species, cultivation of phages with broad host range, an extension of host range of phages, application of phages in combination with EPS-degrading enzymes, genetic modification of phages, or a combination of these strategies. By implementing these strategies, phage therapy could become a new alternative for the prevention and control of drug-resistant multispecies biofilms.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Article

Efficacy of Lytic Phage Cocktails on *Staphylococcus aureus* and *Pseudomonas aeruginosa* in Mixed-Species Planktonic Cultures and Biofilms

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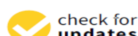


Abstract: The efficacy of phages in multispecies infections has been poorly examined. The *in vitro* lytic efficacies of phage cocktails AB-SA01, AB-PA01, which target *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively, and their combination against their hosts were evaluated in *S. aureus* and *P. aeruginosa* mixed-species planktonic and biofilm cultures. Green fluorescent protein (GFP)-labelled *P. aeruginosa* PAO1 and mCherry-labelled *S. aureus* KUB7 laboratory strains and clinical isolates were used as target bacteria. During real-time monitoring using fluorescence spectrophotometry, the density of mCherry *S. aureus* KUB7 and GFP *P. aeruginosa* PAO1 significantly decreased when treated by their respective phage cocktail, a mixture of phage cocktails, and gentamicin. The decrease in bacterial density measured by relative fluorescence strongly associated with the decline in bacterial cell counts. This microplate-based mixed-species culture treatment monitoring through spectrophotometry combine reproducibility, rapidity, and ease of management. It is amenable to high-throughput screening for phage cocktail efficacy evaluation. Each phage cocktail, the combination of the two phage cocktails, and tetracycline produced significant biofilm biomass reduction in mixed-species biofilms. This study result shows that these phage cocktails lyse their hosts in the presence of non-susceptible bacteria. These data support the use of phage cocktails therapy in infections with multiple bacterial species.

Keywords: phage cocktail therapy; biofilm; planktonic culture; fluorescence; efficacy; mixed-species culture; *Staphylococcus aureus*; *Pseudomonas aeruginosa*

1. Introduction

Wound infections, particularly chronic wounds such as occur in diabetic foot ulcers (DFUs), are often polymicrobial and frequently involve multidrug-resistant (MDR) bacterial pathogens [1,2]. A recent study found that 50% of the bacterial isolates recovered from DFU infections were MDR [3].

Polymicrobial wound infections have reportedly increased in the last decade and include MDR *S. aureus* and *P. aeruginosa,* which are frequently isolated and therapeutically challenging bacteria [4,5]. Both *S. aureus* and *P. aeruginosa* are associated with severe wound infections, including orthopedic infections [4,6], are often isolated together [5], and may occupy different parts of wounds [7]. Co-infections with *S. aureus* and *P. aeruginosa* usually result in worse patient outcomes than infections due to either pathogen alone [8,9]. In addition to their high level of antibiotic resistance, *S. aureus* and *P. aeruginosa* form *in vivo* biofilms that typically result in increased tolerance to antibiotics and contribute to bacterial virulence [8] and immune evasion [10].

Most chronic wound infections involve biofilms, which render antibiotic treatment less effective. Moreover, bacteria within biofilms exhibit altered metabolic properties as compared to planktonic bacteria, which reduces the efficacy of antibiotics in this setting [1,9,11,12]. Phages represent a potential alternative or adjunct therapy for infections with antibiotic-resistant bacteria [13]. However, the efficacy of phages in mixed-species bacterial infections has been incompletely examined, and the limited literature contains conflicting reports [14]. Lytic phages kill their bacterial host by lysis (bursting the infected bacterial cell to release progeny phages) [15]. The process of phage infection and subsequent self-replication in bacteria offers advantages over antibiotics: phages amplify themselves at the infection site provided there are susceptible bacterial hosts [16]. Phages are highly specific to the bacterial species they infect, an advantage over broadly active antimicrobials, as phages are not expected to disrupt a patients' normal microflora. Phages are able to lyse biofilm forms of their host bacteria, such as those typically found in infected DFUs [16,17]. Some studies also suggest that both of these antimicrobial agents in combination are more effective in controlling pathogenic bacteria than either alone [18,19].

Difficulties in growing different bacterial species together *in vitro*, as in the case of *S. aureus* and *P. aeruginosa,* make the study of bacterial interactions and efficacy of antibiotic agents complicated [20,21]. *P. aeruginosa* mostly kills or outcompetes *S. aureus* in *in vitro* co-cultures [22,23]. Medium containing bovine serum albumin (BSA) is recommended to allow better growth of *S. aureus* in the presence of *P. aeruginosa* [24,25]. In this study, we used fluorescence spectrophotometry to evaluate the effectiveness of phage cocktails of *S. aureus* and *P. aeruginosa* under mixed-species planktonic cultures. The use of different fluorescent proteins-labelled bacterial species in mixed-species culture allows monitoring, in real-time, of phage treatment effects based on the detection of a decrease in fluorescence relative to the untreated controls. Fluorescence spectrophotometry is easy to handle, reproducible, and a rapid technique to evaluate treatment efficacy [26,27]. To confirm the spectrophotometry results, we carried out bacterial counts post-treatment, using selective agars. The efficacy of these phage cocktails was also examined in mixed-species biofilms using bacterial counts.

2. Materials and Methods

2.1. Bacterial Species

Clinical isolates of *S. aureus* (n = 4) and *P. aeruginosa* (n = 4) were randomly selected from isolates obtained from South Australia Pathology. These were among the isolates that showed strong susceptibility in spot test and 73–88% biofilm biomass reduction on single-species biofilm experiments because of the phage cocktail and its components treatment (data provided as Supplementary Table S1). Laboratory strains mCherry-labelled *S. aureus* KUB7 [28] and GFP-labelled *P. aeruginosa* PAO1 were generously donated by A/Prof. Heather Jordan of Mississippi State University, USA, and Dr. Nicky Thomas of University of South Australia, Australia, respectively. The fluorescent proteins in the laboratory strains are driven by constitutive promoters and do not need antibiotics to maintain fluorescence expression. Both strains were susceptible to all antibiotics they were exposed during the VITEK® 2 test.

2.2. Phage Cocktails

The phage cocktails AB-SA01 and AB-PA01 were provided by AmpliPhi Biosciences Corporation (now Armata Pharmaceuticals, Inc.) (Los Angeles, CA, USA). AB-SA01 is a combination of three *Myoviridae* staphylococcal phages designated J-Sa-36, Sa-83, and Sa-87. The mean titer, presented in plaque-forming unit/millilitre—PFU/mL, was 9.3 log_{10} (PFU/mL) for J-Sa-36 and Sa-83, 9.0 log_{10} (PFU/mL) for Sa-87, and 9.1 log_{10} (PFU/mL) for the combined product AB-SA01 on *S. aureus* laboratory strains RN4220 and SA6538. AB-PA01 is a combination of Pa-193 and Pa-204 from *Myoviridae*, and Pa-222 and Pa-223 from *Podoviridae*. The titer was 10.5 log_{10} (PFU/mL) for Pa-193, Pa-204, and Pa-222, 9.5 log_{10} (PFU/mL) for Pa-223, and 10.3 log_{10} (PFU/mL) for AB-PA01 on *P. aeruginosa* laboratory strains PAO1 and PA10145. The phage titre was determined using plaque assay, as described [29,30]. None of these phage components encode any known bacterial virulence or antibiotic resistance genes, and all phages were considered to be strictly lytic [29,30]. The phages were produced following the current good manufacturing practice standard (cGMP) and approved by the US Food and Drug Administration as investigational new drugs [30,31].

2.3. Bacterial Identification

The isolates were identified using standard microbiology methods and confirmed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) biotyping (BRUKER Pty. LTD., Melbourne, Victoria, Australia). Antibiotic susceptibility patterns were determined by VITEK® 2 (bioMérieux Australia Pty Ltd., Sydney, New South wales, Australia).

2.4. Mixed-Species Planktonic Cultures and Phage Cocktail Treatment

Mixed-species planktonic cultures were performed following an established protocol [32] with few modifications indicated as follows. In the case of fluorescently-labelled laboratory strains, 2-3 colonies from 18 h selective agar culture plates were suspended in sterile PBS and adjusted to 1.0 McFarland equivalence turbidity standard, containing approximately 8.5 log_{10} (CFU/mL). Each bacterial species suspension was diluted at 1:100 *v/v* with nutrient broth (Sigma-Aldrich, Sydney, New South wales, Australia) supplemented with 5% BSA (Sigma-Aldrich, Sydney, New South wales, Australia) and incubated for 2 h at 37 °C. The two bacterial suspensions were then mixed at the ratio of 1:3 *v/v* of GFP *P. aeruginosa* PAO1 to mCherry *S. aureus* KUB7 in a sterile 10 mL test tube. Two hundred microlitre triplicates of each mixture were transferred to a clear 96-well flat-bottom Greiner CELLSTAR® polystyrene tissue culture plate (Sigma-Aldrich, Sydney, New South wales, Australia), and respective treatments were applied.

Mixed-species planktonic cultures were treated with one of (i) *S. aureus* phage cocktail AB-SA01; (ii) *P. aeruginosa* phage cocktail AB-PA01; (iii) a mixture of the two phage cocktails, AB-SA01+AB-PA01; (iv) gentamicin (positive control); or (v) PBS (negative control). The effect of each phage cocktail and the combination of the two phage cocktails was compared to the gentamicin- and PBS-treated groups.

AB-SA01, AB-PA01, or AB-SA01+AB-PA01 phage cocktails were applied at a multiplicity of infection (MOI) of one to fluorescently labelled *S. aureus-P. aeruginosa* mixed-species culture. Gentamicin was used as a positive control at 16 μg/mL as the minimum inhibitory concentration (MIC) to these isolates was ≤ 8 μg/mL. An equal volume of PBS to phage solutions was applied to negative control groups. A plate cover was applied, and the plate was wrapped with aluminium foil from the top and sides. The plate was incubated in a CLARIOstar Omega plate reader (BMG LABTECH Pty. Ltd., Melbourne, Victoria, Australia) for 24 h at 37 °C with 100 rpm constant double orbital shaking between measurements as described [33] for fluorescent protein-labelled strains. Fluorescence of mCherry and GFP was measured (in relative fluorescence unit, RFU) every 30 min in each well. The excitation and emission wavelengths were set at 570-15 and 620-20 nm for mCherry, and 470-15 and 515-20 nm for GFP detection, respectively. Signals from triplicate wells were averaged and corrected for blank wells containing only nutrient broth. After 24 h incubation, bacterial colony counts were performed

using serial dilution on selective agars for each species, vancomycin-supplemented MacConkey for *P. aeruginosa,* and mannitol salt agar (MSA) for *S. aureus*. The experiment was repeated three times with the same protocol on different days. A similar protocol was followed for clinical isolates, except that incubation was in a standard incubator.

2.5. In Vitro Mixed-Species Biofilm Development and Phage Cocktail Treatment

Mixed-species biofilm development and treatment was conducted using a previously described procedure [34,35] with few modifications. Briefly, 2-3 colonies of 18 h culture of each isolate were independently suspended in sterile PBS and adjusted to 1.0 McFarland turbidity standard. These suspensions were pooled at 1:3 *v/v* ratio of *P. aeruginosa* to *S. aureus*, and 100 µL of the mixed suspension was transferred to 10 mL 5% BSA-nutrient broth. The final suspension was supplemented with 1% sterile glucose to facilitate biofilm development. Two hundred microlitres of the suspension was transferred in triplicate into a tissue culture plate and incubated for 48 h at 37 °C with 70 rpm agitation.

The treatment categories of mixed-species biofilms were (i) *S. aureus* phage cocktail AB-SA01; (ii) *P. aeruginosa* phage cocktail AB-PA01; (iii) a mixture of the two phage cocktails, AB-SA01+AB-PA01; (iv) tetracycline (positive control); and (v) PBS (negative control). The effect of each phage cocktail and a combination of the two phage cocktails was compared to the tetracycline- and PBS-treated groups. Tetracycline (Sigma-Aldrich Corporation, Sydney, New South wales, Australia) was used as a positive control in mixed-species biofilm treatment as it was strongly effective ($p < 0.001$), compared with PBS treatment, in biofilm biomass reduction on single-species biofilm treatment of both *S. aureus* and *P. aeruginosa* isolates. However, gentamicin did not produce significant biofilm biomass reduction ($p > 0.05$), compared with PBS treatment (Unpublished data). Tetracycline was not used as a positive control in mixed-species planktonic culture treatment experiments to avoid exaggerated fluorescence detection because of its color and fluorescent nature [36].

Next, the liquid culture was removed, and plates were washed gently twice using sterile deionized water. Then, 225 µL of AB-SA01, AB-PA01, or AB-SA01+AB-PA01 in the nutrient broth was applied to the respective treatment group biofilms. An equivalent volume of tetracycline and PBS to phage solution in nutrient broth were also applied as controls. The concentration of tetracycline was 128 µg/mL because *P. aeruginosa* isolates are susceptible to a higher concentration of tetracycline [37,38]. The MIC of tetracycline for *S. aureus* was ≤ 8 µg/mL during VITEK® 2 antimicrobial susceptibility test. Treated biofilms were incubated for 12 h at 37 °C with no agitation. The biofilm was washed twice using 250 µL sterile PBS through careful pipetting. The biofilm-associated cells attached to the well surface were collected with 225 µL nutrient broth by pipetting after scraping the surface with a loop as described earlier [39,40]. After homogenization with a vortex mixer, the cell suspension was serially diluted, 10^{-1}–10^{-8}, in filter-sterilized 10 mM ferrous ammonium sulphate (FAS) supplemented nutrient broth to inactivate free phage [41] and incubated at room temperature for 15 min.

2.6. Viable Bacterial Cell Count

One hundred microlitres of the bacterial suspension from each serial dilution was then mixed with 3 mL nutrient soft agar warmed at 42 °C and dispensed over 37 °C pre-warmed MSA and vancomycin-supplemented MacConkey agar in triplicate and incubated at 37 °C for 24 h. Plates with approximately 30–300 colonies were taken from one of the dilutions, and colony count was carried out as previously described [42]. The bacterial cell count was calculated using the formula $B = N/d$ where B = number of bacteria; N = average number of colonies counted on three plates; d = dilution factor as described earlier [43]. The results are expressed as logarithm-transformed values (log (CFU/mL)).

2.7. Statistical Analysis

STATA version 16 software was used for statistical analysis. Data are reported in terms of the mean. A comparison of experimental groups was performed using a one-way analysis of variance (two-tailed) or paired '*t-test*'. A $p < 0.05$ value was considered statistically significant.

3. Results

3.1. *Effect of Phage Cocktails on Fluorescently Labelled Mixed-Species Planktonic Cultures*

The fluorescence of each species alone or together was confirmed under a confocal microscope, as shown in Figure 1.

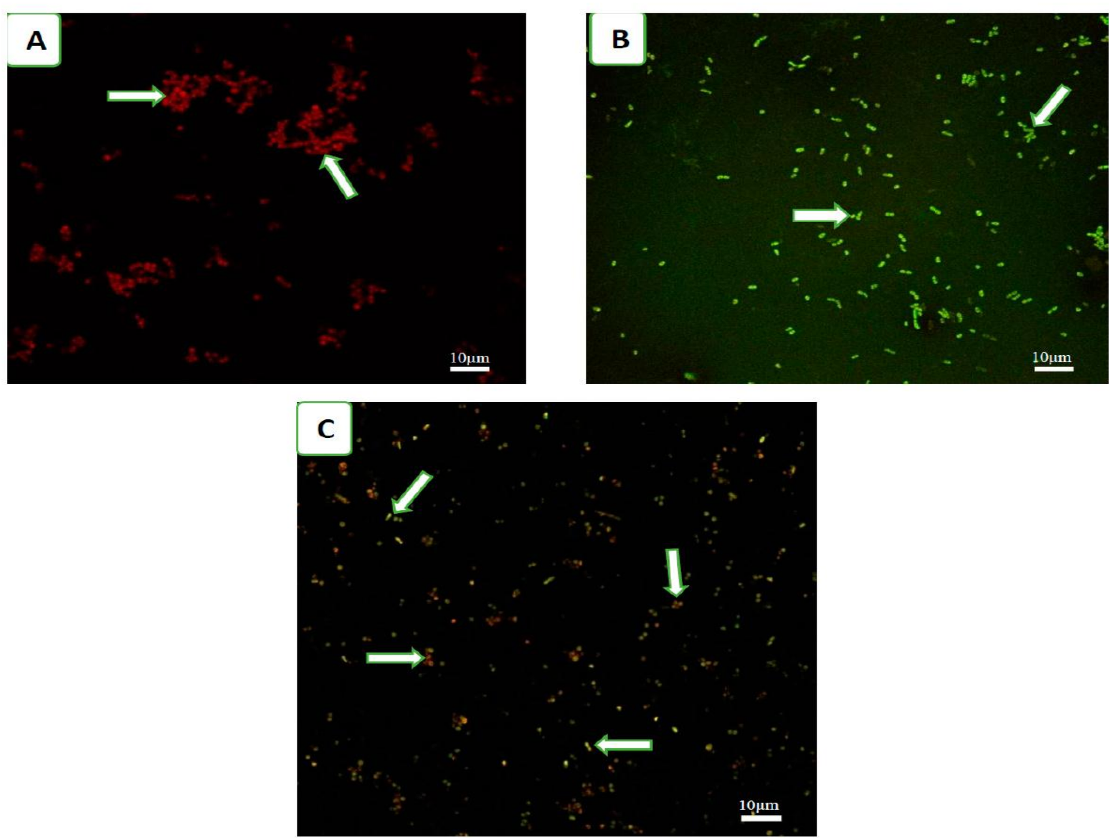
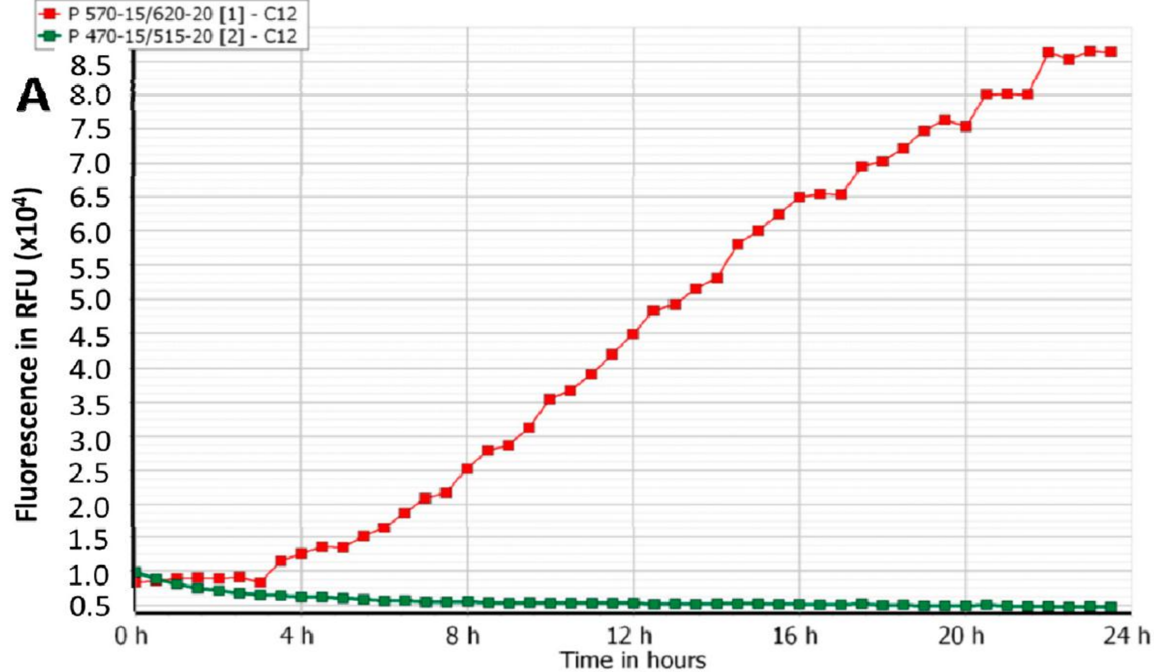


Figure 1. Fluorescence strain under confocal microscopy: (**A**) mCherry-labelled *S. aureus* KUB7, (**B**) GFP-labelled *P. aeruginosa* PAO1, and (**C**) mCherry-labelled *S. aureus* KUB7 mixed with GFP-labelled *P. aeruginosa* PAO1.

The growth of fluorescently-labelled *S. aureus* and *P. aeruginosa* in single- and mixed-species planktonic cultures in the presence and absence of phages is shown in Figure 2A–G. Compared to the phosphate-buffered saline (PBS) treatment, AB-SA01 and AB-PA01 treatments effectively halted the growth of their host throughout the 24 h follow-up period. In the single-species cultures treated with PBS, there was a marked increase in fluorescence of mCherry *S. aureus* KUB7 and GFP *P. aeruginosa* PAO1, as shown in Figure 2A,B, indicating bacterial growth. In the mixed-species cultures without phages, the magnitude of fluorescence slowly increased with time (Figure 2C). However, the maximum red and green fluorescence obtained was much lower than the fluorescence detected during single-species PBS-treated cultures (Figure 2A,B), suggesting that the mixed-species exhibited competition for nutrients or co-inhibitory effects. In mixed-species cultures treated with a single phage cocktail, the fluorescence of the target host was almost eliminated, while the non-target host was unaffected, as shown in Figure 2D,E, with fluorescence similar to PBS-treated single-species control.



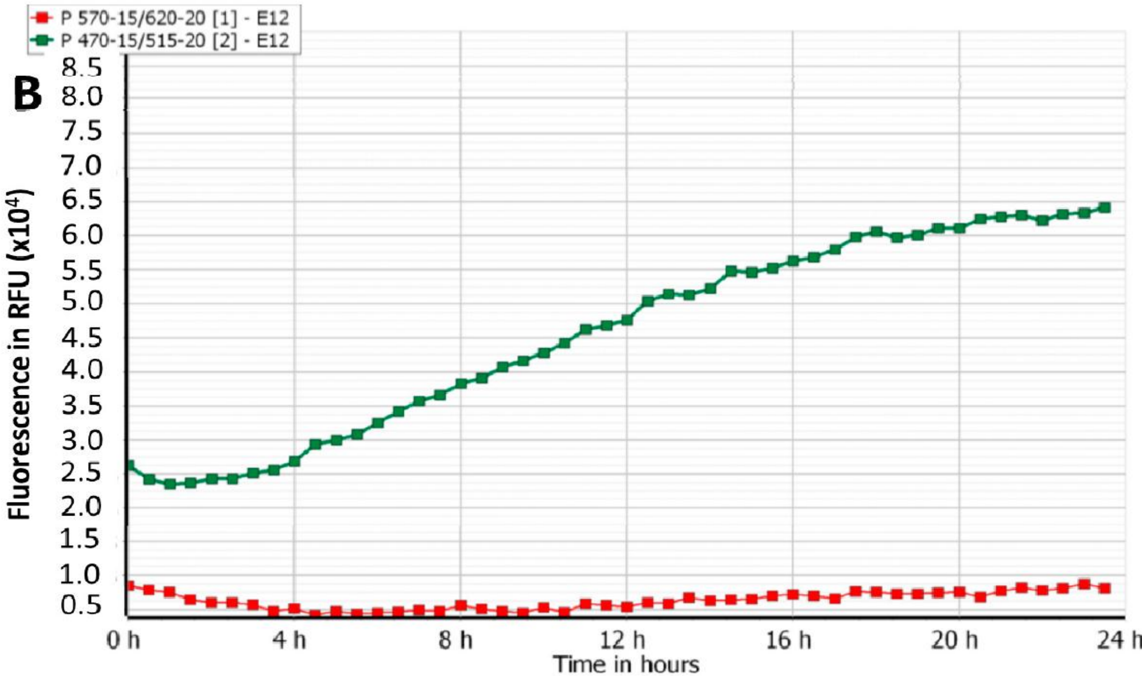
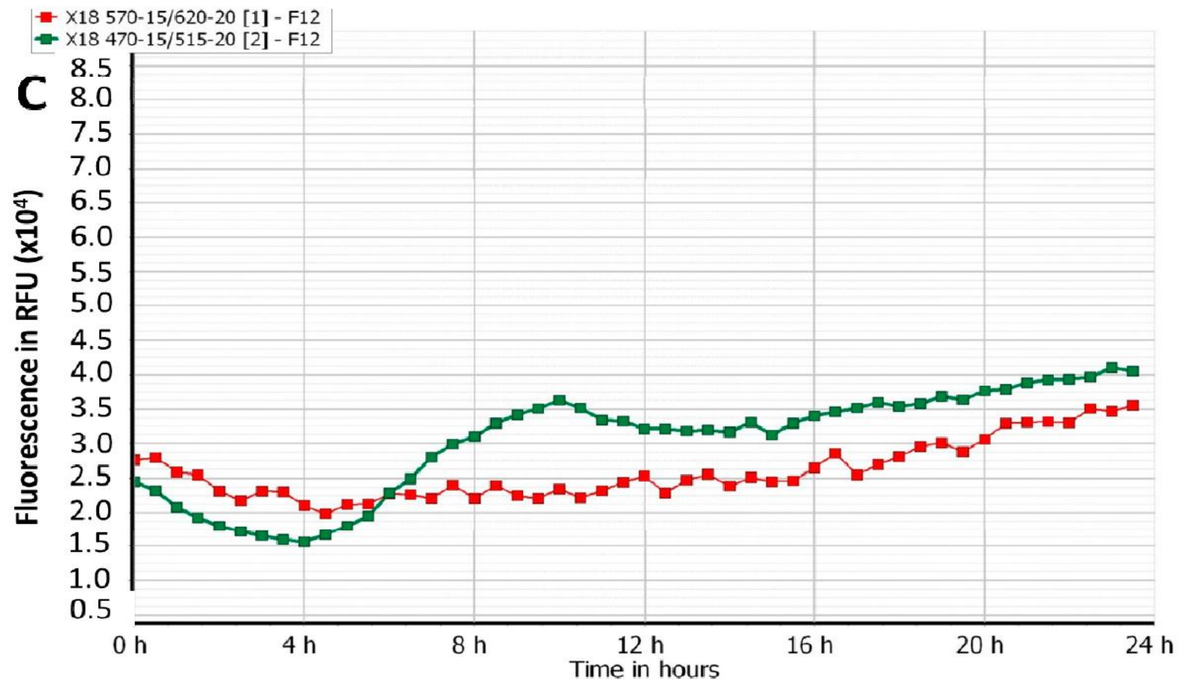


Figure 2. *Cont.*



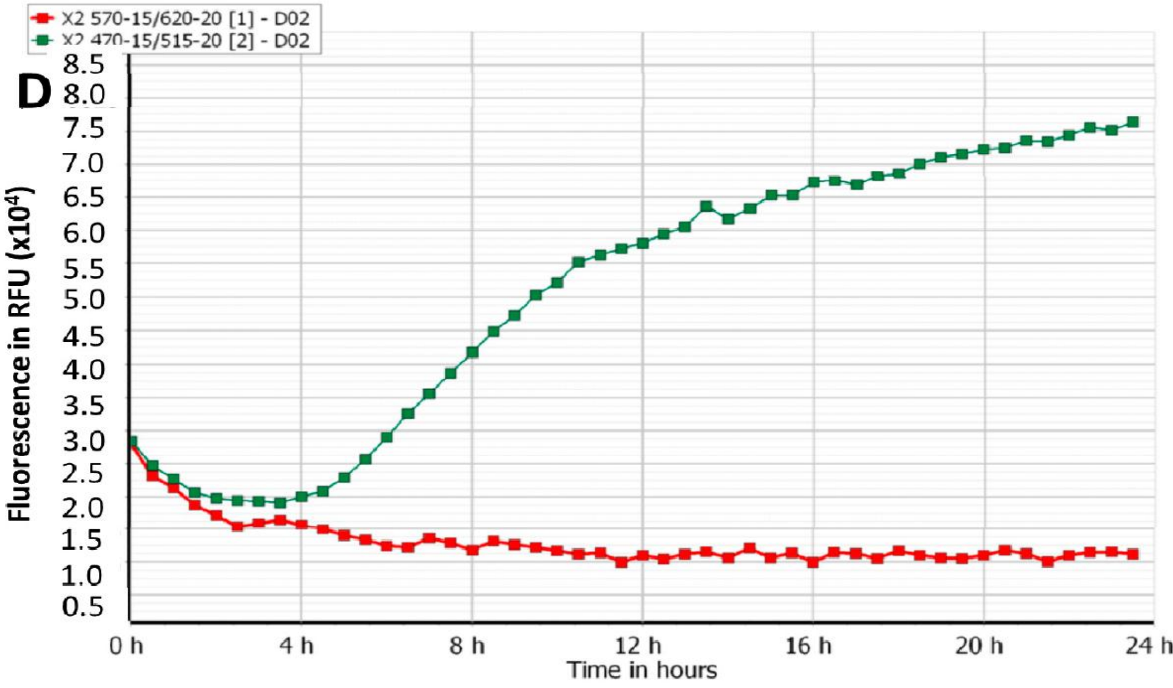
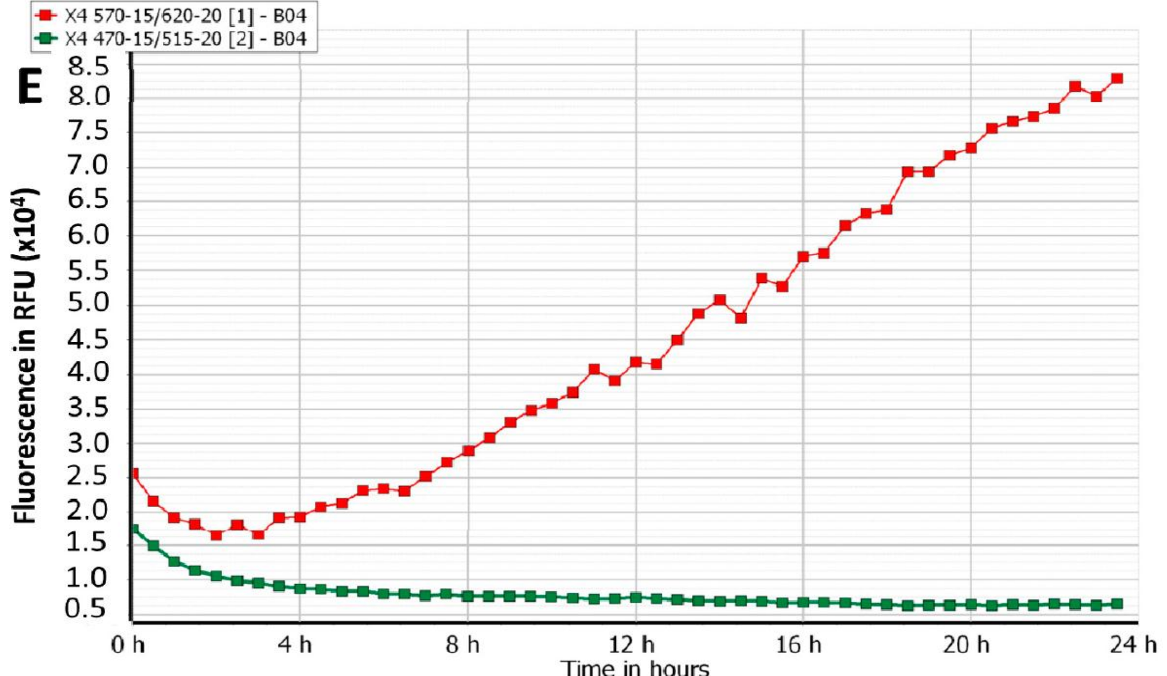


Figure 2. *Cont.*



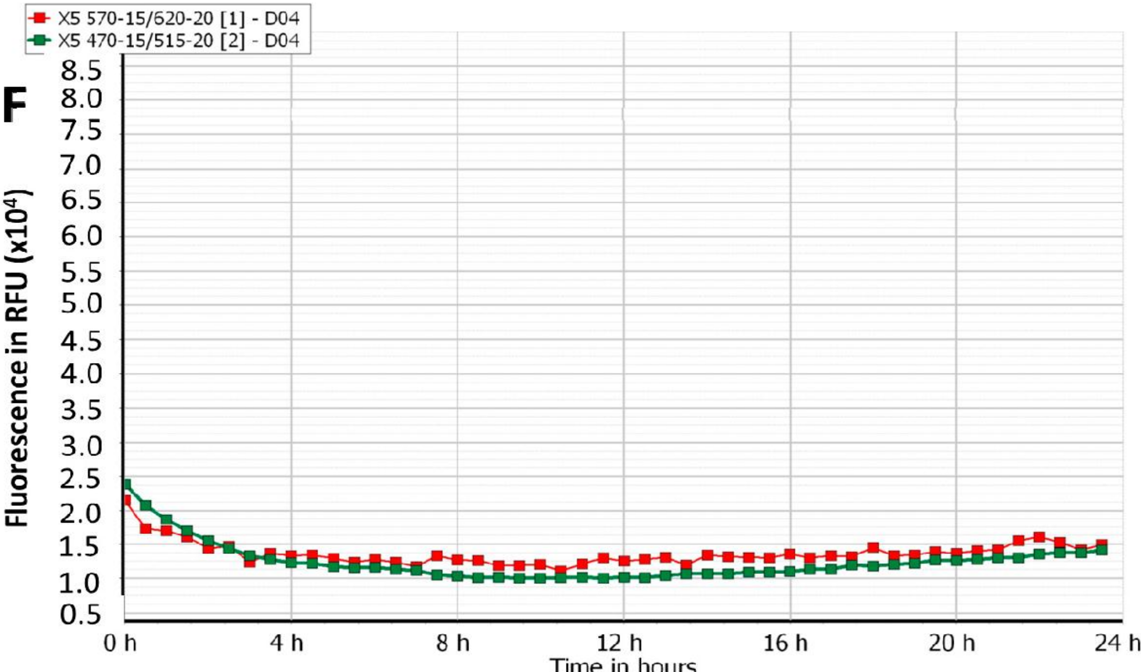


Figure 2. *Cont.*

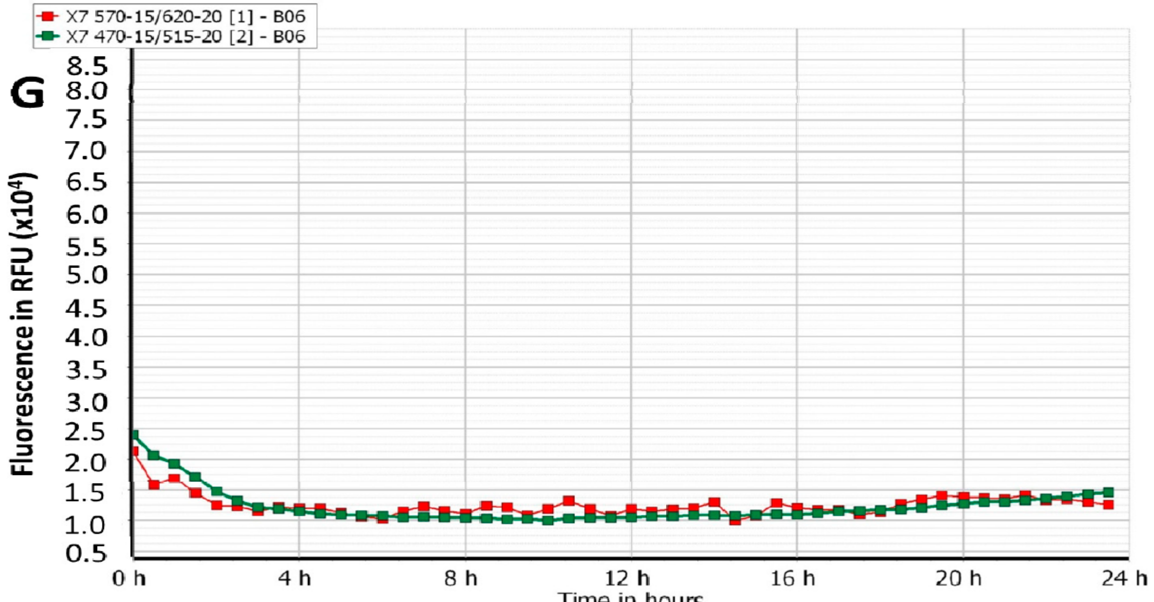


Figure 2. **A–G:** effect of phage cocktails on mCherry *S. aureus* KUB7 (red data points) and GFP *P. aeruginosa* PAO1 (green data points) single- and mixed-species planktonic cultures. RFU represents relative fluorescence unit. (**A**) PBS-treated mCherry *S. aureus* KUB7 (in red) single-species culture; the green graph is due to background detection since the machine was set for mCherry and GFP detection. (**B**) PBS-treated GFP *P. aeruginosa* PAO1 (in green) single-species culture; the red graph is due to background detection since the machine was set for mCherry and GFP detection. (**C**) PBS-treated mCherry *S. aureus* KUB7 and GFP *P. aeruginosa* PAO1 mixed-species culture. (**D**) AB-SA01-treated mCherry *S. aureus* KUB7 and GFP *P. aeruginosa* PAO1 mixed-species culture. (**E**) AB-PA01-treated mCherry *S. aureus* KUB7 and GFP *P. aeruginosa* PAO1 mixed-species culture. (**F**) AB-PA01+AB-SA01-treated mCherry *S. aureus* KUB7 and GFP *P. aeruginosa* PAO1 mixed-species culture. (**G**) Gentamicin-treated mCherry *S. aureus* KUB7 and GFP *P. aeruginosa* PAO1 mixed-species culture.

When both phage cocktails were added to the mixed-species cultures, there was low fluorescence of both bacterial species, as shown in Figure 2F, similar to the inhibitory effect of gentamicin (Figure 2G), indicating that phage efficacy is not affected by the presence of non-host bacteria or other phages. The highest magnitude of red fluorescence in mCherry *S. aureus* KUB7 was detected from the untreated single-species culture (Figure 2A). In the case of GFP *P. aeruginosa* PAO1, the highest green fluorescence was detected from mixed-species culture treated with *S. aureus* phage cocktail, AB-SA01 (Figure 2D). The lowest magnitude of fluorescence from the target host in the mixed-species culture was observed when treated with each phage cocktail, as shown in Figure 2D,E. As expected, AB-SA01 and AB-PA01 exhibited no lytic effect on non-susceptible hosts (Figure 2D,E). While the fluorescence detected from a non-susceptible host showed an increase through time, the fluorescence obtained from the susceptible host remained low.

The decreases in fluorescence from each phage cocktail-, combinations of the two phage cocktails-, and gentamicin-treated groups were significantly lower compared to the PBS-treated group. The corresponding colony count results for each treatment group after 24 h are shown in Table 1 and confirm the results obtained with the fluorescence detection methodology.

Table 1. Bacteria cell count (log_{10} (CFU/mL)) of *S. aureus* and *P. aeruginosa* in mixed-species planktonic culture after 24 h phage cocktails, gentamicin, or PBS treatment.

Isolates Combination	Evaluated Isolate	Bacterial Cell Counts after Treatment				
		PBS	**AB-SA01**	**AB-PA01**	**AB-SA01+ AB-PA01**	**Gentamicin**
S. aureus KUB7 [S] and GFP PAO1 [P]	*S. aureus* KUB7 [S]	5.5	3.6	8.7	3.0	0
	GFP PAO1 [P]	7.9	8.9	0	3.7	0
63-6538 [S] and 63-6598 [P]	63-6538 [S]	5.6	1.5	7.9	0	0
	63-6598 [P]	5.3	6.0	0	0	0
63-2498 [S] and 63-5497 [P]	63-2498 [S]	6.2	0	6.6	0	0
	63-5497 [P]	7.5	8.0	3.5	4.0	0
63-5656 [S] and 63-6036 [P]	63-5656 [S]	4.8	3.6	6.1	0	0
	63-6036 [P]	6.6	8.1	3.3	4.2	0
Summarized treatment effect						
	S. aureus mean	5.5	2.2	7.3	0.8	0
	S. aureus reduction	—	3.3	+1.8	4.7	5.5
	P. aeruginosa mean	6.8	7.8	1.7	3.0	0
	P. aeruginosa reduction	—	+1.0	5.1	3.8	6.8

[S] *S. aureus* isolates, [P] *P. aeruginosa* isolates, + indicates an increase in bacterial count compared to PBS treatment.

3.2. *Efficacy of Phage Cocktails on Laboratory and Clinical Isolates Mixed-Species Planktonic Cultures*

The population of each bacterial species in mixed-species planktonic cultures at the end of 24 h of treatment was assessed. Compared to PBS-treated samples, AB-SA01- and AB-PA01-treated samples produced 3.3 log_{10} (CFU/mL) and 5.1 log_{10} (CFU/mL) reduction on their hosts, respectively, as shown in Table 1. These reductions in bacterial cell count were associated with the susceptibility of each bacterial isolates to the specific phage cocktail and its component phages during spot test (see complementary data, Table S1). When the same samples were treated with the combination of the two phage cocktails, AB-SA01+AB-PA01, the mean cell count of *S. aureus* and *P. aeruginosa* reduced by 4.7 log_{10} (CFU/mL) and 3.8 log_{10} (CFU/mL), respectively. The cell counts of one bacterial species showed an increase when the culture was treated with only a phage cocktail of the other species in the mixed-species culture. All planktonic cultures treated with gentamicin yielded no viable bacterial cells.

3.3. *Effect of Phage Cocktails Treatment on Mixed-Species Biofilms*

The findings of this study demonstrate that phage cocktails AB-SA01, AB-PA01, and a mixture of AB-SA01 and AB-PA01 successfully lysed their hosts in the presence of biofilms of non-susceptible species. These phage cocktails applied to *S. aureus* and *P. aeruginosa* mixed-species biofilms caused a statistically significant ($p < 0.05$; Table 2) reduction in the host cell population compared to the PBS-treated group, as shown in Table 2. However, the reduction of the cell population in *S. aureus* and *P. aeruginosa* was less than half of the decrease observed in planktonic culture treatment. Most of the tetracycline-treated cultures produced no or the lowest number of bacterial cells.

Compared to PBS treatment, the application of AB-SA01 or AB-PA01 alone did not produce a statistically significant effect on the cell count of the non-host bacterial species population ($p > 0.05$; 6.9 vs. 6.8 for AB-SA01 and 6.2 vs. 5.8 for AB-PA01, Table 2). The mean bacterial cell population of each species remained unaffected when treated with the other species' phage cocktail alone. Treatment of mixed-species biofilms using the mixture of the two phage cocktails, AB-SA01+AB-PA01, produced similar cell reduction on both *S. aureus* and *P. aeruginosa* isolates as each phage cocktail treatment. The effect of tetracycline treatment caused a significant reduction in the population of both bacterial species.

Table 2. Bacteria count (log_{10} (CFU/mL)) of *S. aureus* and *P. aeruginosa* in mixed-species biofilms after 24 h phage cocktails, tetracycline, and PBS treatment.

Isolates Combination	Evaluated Isolate	Bacterial Cell Counts after Treatment				
		PBS	AB-SA01	AB-PA01	AB-SA01+ AB-PA01	Tetracycline
S. aureus KUB7 [S] and PAO1 GFP [P]	*S. aureus* KUB7 [S]	6.2	4.5	7.4	5.5	3.8
	PAO1 GFP [P]	6.4	6.3	3.8	4.0	3.9
63-6538 [S] and 63-6598 [P]	63-6538 [S]	5.2	4.4	4.7	3.6	3.0
	63-6598 [P]	5.5	5.6	3.6	3.7	0
63-2498 [S] and 63-5497 [P]	63-2498 [S]	6.3	4.4	5.2	5.5	0
	63-5497 [P]	7.1	6.2	5.5	5.2	0
63-5656[S] and 63-6036[P]	63-5656 [S]	7	4.9	5.9	5.3	0
	63-6036 [P]	8.5	8.9	4.7	6.3	0
Summarized treatment effect						
	S. aureus mean	6.2	4.6	5.8	5.0	1.7
	S. aureus reduction	—-	1.6	0.4	1.2	4.5
	P. aeruginosa mean	6.9	6.8	4.4	4.8	1.0
	P. aeruginosa reduction	—-	0.1	2.5	2.1	5.9

[S] *S. aureus* isolates, [P] *P. aeruginosa* isolates.

4. Discussion

The rationale to examine the effect of phage treatment on mixed-species planktonic and biofilm cultures was that many wound infections are polymicrobial and contain bacteria in biofilm forms [44] and that there is a paucity of data on the action of phages under such circumstances. In polymicrobial infections, there exist interspecies interactions, ranging from antagonism to cooperation, that can significantly impact the pathogenicity of microbes and clinical outcomes of infections [45]. Examination of the bacterial population using fluorescence detection and CFU count results suggest that phage cocktail treatment is effective both in planktonic and biofilm states of the host bacteria under mixed-species cultures.

Multiple fluorescent proteins can be simultaneously applied to examine different microbial populations in real-time [46,47]. The use of mCherry in combination with GFP is suitable as the excitation and emission spectra of these proteins are well separated [46,48]. The advantages of using mCherry and GFP as markers include ease of detection, no exogenous substrate is required that may perturb biological samples, no need for cell processing for visualization, and they are suitable for real-time monitoring of cells in mixed cultures [49,50]. In this study, the high magnitude of fluorescence detected and the bacterial cell population obtained during single species culture without treatment show bacterial growth and fitness capability for the model while fluorescing [26,49–51]. Hence, we used mCherry *S. aureus* KUB7 and GFP *P. aeruginosa* PAO1 to distinguish them in mixed-species culture. In the current study, loss of mCherry or GFP fluorescence was considered an indication of bacterial death due to phage-induced lysis, which was supported by decreased or no CFU. Previous studies also showed that the decrease in fluorescence is due to cell death, suggesting it to be an early and sensitive marker of viability [26,52].

There was a strong association between the final bacterial density reading through fluorescence detection and bacterial cell population data as measured by colony count. For both mCherry *S. aureus* KUB7 and GFP *P. aeruginosa* PAO1, the fluorescence signal increased for PBS-treated or bacterial species unaffected by the treatment. The higher green fluorescence detected in AB-SA01-treated mixed-species culture than in GFP P. *aeruginosa* PAO1 single-species PBS-treated culture might be attributed to more pronounced *P. aeruginosa* population growth in the absence of competing organism or enhanced growth because of accessibility to more nutrients such as iron from the dead *S. aureus* cells [53]. The magnitude of fluorescence of mCherry or GFP obtained was minimal when the mixed-species

bacterial cultures were treated with each phage cocktail alone. The fluorescence magnitude and bacterial cell population obtained during AB-SA01+AB-PA01 and gentamicin treatments were similar; the fluorescence records are low; the fluorescence graphs are overlapped and remained at their lower levels throughout the experiment period, and bacterial population recovered at the end of the experiment is minimal or none. This finding shows that treatment with each phage cocktails separately or in combination produced a similar effect to gentamicin treatment in planktonic mixed-species culture. Our experiments also demonstrated that bacterial density could be estimated, and the treatment effect evaluated in mixed-species cultures through fluorescence spectrophotometry using different fluorescent proteins-labelled bacterial species.

The magnitude of fluorescence detected in the untreated mixed-species culture was lower compared to untreated single-species cultures, which might be attributable to competition between the two bacterial species [21–23]. All these decreased or increased fluorescence detected were strongly associated with the decreased or increased colony count results, respectively. Our observations support previous reports that show the magnitude of fluorescence, absorbance, and colony count results are supplementary to one another in characterizing bacterial growth [54,55].

The magnitudes of fluorescence obtained from the three replica wells across the three independent experiments on different days with the same protocol were similar, demonstrating reliability of the method and reproducibility of results. The decrease in fluorescence of treated samples is consistent with efficient bacterial cell lysis by the host-specific phage cocktail as measured through colony count. This result agrees with a study that compared the fluorescence with a CFU count method in single-species culture [26]. In the mixed-species planktonic cultures of clinical isolates, treatment with each phage cocktail alone caused a significant ($p < 0.001$) decline of the target bacterial host population. Treatment with a combination of AB-SA01 and AB-PA01 also resulted in a significant ($p < 0.05$) decrease in cell density of both bacterial species. Our findings are similar to a study that showed planktonic *E. coli* grown in co-culture with *Salmonella* enterica did not survive attack from *E. coli* specific phages [56].

Mixed-species biofilms are complex communities that affect the physiological state of host bacterial cells and the availability of phage receptors, possibly due to competition with other bacterial species [14,39]. Our findings of mixed-species biofilm treatment show that the effect of both phage cocktails separately and in combination, AB-SA01+AB-PA01, significantly ($p \leq 0.05$) reduced the target bacterial host population. The effect of these phage cocktails was lower in biofilm than in planktonic states of their hosts, which agrees with previous reports [35,39,57–59]. Possible explanations include: the complex extracellular biofilm matrix may reduce the efficacy of phages because of entrapment of phage particles [57], reduced multiplication of phages due to the large proportion of metabolically inactive host cells in biofilms [60], or shedding of phage receptors from the host bacteria in biofilms [61]. It might also be partly explained by the fact that dead cells resulting from phage attack might support surviving bacteria through serving as a nutrient reservoir and providing a shield from phage attack by phage binding to receptors on dead bacteria [62]. Tetracycline treatment showed superior bacterial population reduction, on both bacterial species, compared to the phage cocktail formulations used in this study. Our finding is consistent with a previous report that showed the application of phiIPLA-RODI, phiIPLA-C1C, and the combination of the two phages is more efficient in the planktonic phase than that in biofilms phase during *S. aureus* IPLA16 and *S. epidermidis* LO5081 mixed-species cultures [39].

In this study, we noted that phage cocktails applied to mixed-species biofilms could effectively reduce bacterial host populations. We did not observe the protection of susceptible bacterial hosts from phage attack in mixed-species biofilms by non-susceptible bacteria. This observation is consistent with some studies [34,40]. However, it is in contrast with a previous phage treatment study that concluded the structural heterogeneity of the biofilm from mixed-species produced pockets of unreachable susceptible bacteria [63]. Because we found significant bacterial host cell reduction during each

phage cocktail treatment in mixed-species biofilm, protection from lysis for the target bacteria by the non-susceptible bacterial host cannot be assumed [40].

Our bacterial count data are in line with previous findings of biofilms infected with phages for 24 h and more extended periods [63,64]. Overall, our results confirm that lytic phages can be efficient in mixed-species planktonic and biofilm states. This study also demonstrates the feasibility of *in vitro* real-time monitoring of the efficacy of phage treatments using fluorescently labelled bacteria in mixed-species cultures through spectrophotometry, which is a simple, rapid, and reliable procedure.

5. Conclusions

Our findings suggest that the use of phage cocktails in mixed-species planktonic or biofilm state could provide practical alternatives to antibiotics in combating antibiotic-resistant infections. The association between the decrease or loss of fluorescence during real-time monitoring of the effect of phage cocktails with the decrease in numbers of bacterial cells as measured by colony counts, across the three replicas and three experiments in different days with the same protocol, shows the effectiveness of the phage cocktail treatments *in vitro* and repeatability of the results. The present study findings show that phages can reduce the host bacterial cell population significantly from planktonic and biofilm states. The lytic efficacy of AB-SA01, AB-PA01, and their combination on antibiotic-resistant bacterial hosts in mixed-species planktonic and biofilm phases is a clear indication of the potential of phages to mitigate antibiotic-resistant infections.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/5/559/s1, Table S1: Phage cocktail and component phages susceptibility and biofilm reduction (in percentage) after phage cocktail treatment of the selected bacterial isolates

Author Contributions: L.G.K. participated in the conception of the research idea and design of the methodology, carried out laboratory experiment and data collection, and prepared the manuscript. P.G.S. and J.G.M. participated in the conception of the research idea and design of the methodology. P.G.S. and L.G.K. performed data analysis. M.S.W., S.M., N.T., D.L.G., J.G.M., and P.G.S. edited the manuscript. All researchers approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: S.M was an AmpliPhi Biosciences employee at the time of performing the experiments and contributed to the scientific discussion. All other authors declare no conflict of interest.

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Table 1: Susceptibility of *P. aeruginosa* isolates to phage cocktail AB-PA01 and its components

		Phages				
No.	Isolates ID	Pa 193	Pa 204	Pa 222	Pa 223	AB-PA01
1.	62-8241A	S	R	I	R	I
2.	48489280B	S	S	S	S	S
3.	49540983	S	S	S	S	S
4.	49669841A	S	I	S	S	S
5.	63-8601	S	S	S	S	S
6.	63-5497	S	S	S	S	S
7.	63-8199	S	I	S	S	S
8.	P04-5393	S	S	S	S	S
9.	63-7648	S	R	S	R	S
10.	63-6608	R	I	S	I	S
11.	62-8028A	I	R	I	R	I
12.	62-8001	I	R	S	S	S
13.	63-3521	S	I	I	I	S
14.	63-7889	S	S	S	S	S
15.	63-3624	R	R	R	R	R
16.	63-7651	S	S	S	I	S
17.	63-7780	S	S	S	S	S
18.	63-5405	S	R	R	R	I
19.	49669854	S	S	S	S	S
20.	63-7871	S	R	S	I	S
21.	63-7996	R	R	S	R	I
22.	63-8795	R	S	R	S	S
23.	63-8788	S	R	R	S	R
24.	63-9029	R	I	S	S	S
25.	63-2220	S	I	S	R	S

26.	S-63-5289	S	S	I	S	S
27.	62-5656	I	I	S	I	S
28.	63-6728	S	S	S	I	S
29.	63-5713	S	S	S	S	S
30.	63-296P	S	R	S	R	S
31.	04-5755P	S	R	I	R	S
32.	63-9513	S	S	S	S	S
33.	63-6036	S	S	S	S	S
34.	8882-P	S	I	R	I	I
35.	8782-P	S	S	S	S	S
36.	63-6598	S	R	S	R	S
37.	P63-7786	S	R	S	R	S
38.	63-6299	S	S	S	S	S
39.	PA01GFP	S	S	S	S	S
40.	PA10145 FU	S	S	S	S	S
41.	PA15692	S	S	S	S	S

Key: S = susceptible, R = resistance, and I = intermediate

Table 2: Susceptibility of *S. aureus* isolates to phage cocktail AB-SA01 and its components

No	Bacterial ID	Phages			
		J-Sa 36	Sa 83	Sa 87	Cocktail
1.	SA4220 mCherry	S	S	S	S
2.	SA6538	S	S	S	S
3.	04-5755 Staph	R	I	S	R
4.	63-6354	S	I	S	S
5.	46834854	I	I	I	R
6.	63-2498	S	S	S	S
7.	63-6758	S	S	S	S
8.	63-6126	S	S	S	S
9.	63-169	S	S	S	S
10.	63-7647	R	R	S	S
11.	63-7148	S	S	S	S
12.	48556348	S	S	S	S
13.	62-9282	R	R	R	R
14.	60271003	S	S	S	S
15.	63-5879	S	S	S	S
16.	63-2749	I	S	S	S
17.	49639617	R	R	I	R
18.	49029122	S	S	S	S
19.	48561341	R	R	S	S
20.	60262911	S	S	S	S
21.	63-145	R	R	R	R
22.	63-2599	S	S	S	S
23.	63-3395	R	S	S	S
24.	63-5402	R	I	I	I
25.	63-5273	S	S	I	S
26.	63-5656	S	S	S	S
27.	O4-5089	R	R	S	I
28.	63-7334	S	S	S	S
29.	41535062	R	S	S	S
30.	90271071	S	S	S	S
31.	48392361	I	I	S	S
32.	62-8600	I	R	R	S
33.	62-9187	R	R	I	R

34.	63-2482	S	S	S	S
35.	63-3485	I	S	S	S
36.	63-5612	S	S	S	S
37.	63-6352	S	S	S	S
38.	63-6832	S	S	S	S
39.	O4-5216	S	S	S	S
40.	63-8782S	S	I	S	S
41.	47597332	S	S	S	S
42.	99279922	R	I	I	S
43.	93952969	I	I	S	S
44.	62-8701	S	S	S	S
45.	63-2548	S	S	S	S
46.	63-3534	S	S	S	S
47.	63-5339	R	R	S	S
48.	63-5222	S	S	S	S
49.	63-5853	S	S	S	S
50.	63-6378	S	S	S	S
51.	63-7019	S	S	S	S
52.	63-7252	S	S	S	S
53.	63-6036	S	S	S	S

Key: S = susceptible, R = resistance, and I = intermediate

Table 3: Effect of phage cocktail AB-SA01 on *S. aureus* biofilm biomass

No.	Isolates ID	OD reading after treatment		Difference between phage and PBS treated (percentage)
		Phage treated	PBS treated	
1.	SA4220mCh	0.14	0.29	51.72%
2.	SA6538	0.08	0.96	91.66%
3.	63-5897	0.27	0.54	50%
4.	48561341	0.31	0.46	32.60%
5.	63-6378	0.03	0.16	81.25%
6.	63-7148	0.19	0.29	34.48%
7.	63-2498	0.04	0.2	80.00%
8.	48556348	0.06	0.13	53.85%
9.	48556348	0.04	0.06	33.33%
10.	63-6758	0.04	0.15	73.33%
11.	60271003	0.1	0.19	47.36%
12.	63-7647	0.26	0.35	25.71%
13.	63-2482	0.08	0.21	61.90%
14.	41535062	0.07	0.16	56.25%
15.	63-2599Staph	0.21	0.95	77.89%
16.	63-6036	0.07	0.25	72.00%
17.	63-5656	0.09	0.37	75.68%
18.	63952969	0.06	0.22	72.72%
19.	63-3534	0.06	0.19	68.42%
20.	47597332	0.06	0.19	68.50%
21.	62-8600	0.06	0.27	77.78%
22.	48392361	0.73	0.96	23.96%
23.	62-8701	0.08	0.26	69.23%
24.	63-5339	0.06	0.2	70%
25.	62-5222	0.05	0.22	77.27%

26.	63-2548	0.06	0.19	68.42%
27.	63-5612	0.06	0.21	71.43%
28.	63-7019	0.06	0.26	76.92%
29.	63-5853	0.05	0.21	76.19%
30.	63-6832	0.16	0.47	65.96%
31.	63-5273	0.74	1.82	59.34%
32.	63-169	0.13	0.34	61.76%
33.	60262911	0.09	0.45	80.00%
34.	99279922	0.22	0.26	15.38%
35.	Sa04-5089	0.15	0.19	21.05%
36.	90271071	0.09	0.13	30.77%
37.	63-7334	0.06	0.13	53.84%
38.	63-5402	0.16	0.18	11.11%
39.	63-145	0.13	0.14	7.14%
40.	49639617	0.18	0.2	10%
41.	62-9187	0.14	0.14	0.00%
42.	Sa04-5216	0.07	0.24	70.83%
43.	63-3395	0.07	0.24	70.83%
44.	63-3485	0.1	0.2	50.00%
45.	63-6352	0.99	0.15	P84.85%
46.	63-8782S	0.13	0.23	43.48%
47.	63-7252	0.07	0.28	75%
48.	63-2749	0.15	0.21	28.57%
49.	62-9282	0.22	0.25	12%
50.	46834854	0.2	0.22	9.09%
51.	Sa04-5755	0.16	0.16	0.00%
52.	63-6354	0.31	0.39	+20.5%
53.	63-6126	0.39	0.48	+18.75%

Table 4: Effect of phage cocktail AB-PA01 on *P. aeruginosa* biofilm biomass

No.	ID	Phage Treated	Not Phage treated	Difference in %
1.	63-5405	1.24	1.29	3.88%
2.	63-8601	0.16	0.17	5.88%
3.	62-2220	1.1	1.29	14.73%
4.	63-8788	2.29	2.75	16.73%
5.	63-9029	0.9	1.18	23.73%
6.	63-7651	1.45	1.99	27.14%
7.	48489280B	1.2	1.7	29.41%
8.	62-8241A	0.91	1.42	35.92%
9.	63-8795	1.98	3.14	36.94%
10.	63-7786	1.44	2.34	38.46%
11.	63-7871	2.26	3.7	38.92%
12.	63-5656	1.13	2.14	47.20%
13.	49540983	0.85	1.67	49.10%
14.	63-6608	1.15	2.36	51.27%
15.	PA15692	0.16	0.33	51.51%
16.	49669854	0.93	1.92	51.56%
17.	62-8001	0.68	1.42	52.11%
18.	63-7996	0.86	1.83	53.01%
19.	PAO1 GFP	0.24	0.55	56.36%
20.	63-6299	0.97	2.36	58.90%
21.	63-6728	0.96	2.41	60.02%
22.	63-3521	0.33	0.85	61.18%
23.	PA04-5755	0.72	2.1	65.71%
24.	PA04-5393	0.63	2	68.50%
25.	63-5713	0.52	1.81	71.27%
26.	PA10145FU	0.44	1.58	72.15%

27.	63-296 P	0.5	1.83	72.68%
28.	63-6036	0.49	1.81	72.78%
29.	63-9513	0.36	1.33	72.93%
30.	63-8199	0.33	1.29	74.42%
31.	49669841A	0.22	0.99	77.78%
32.	63-7889	0.17	0.78	78.21%
33.	63-5497	0.34	1.76	80.68%
34.	62-8028A	0.15	0.81	81.48%
35.	63-8782P	0.33	1.83	81.97%
36.	63-7780	0.04	0.3	86.87%
37.	63-6598	0.12	1.02	88.24%
38.	63-3624	1.56	1.41	+10.64%
39.	63-8882P	1.29	1.06	+21.70%
40.	63-7648	1.68	1.33	+26.32%

Table 5: Comparison of effect of phage and tetracycline (TTC) treatment on *P. aeruginosa* biofilm reduction

Isolates ID	OD measurement after: Phage treatment	PBS treatment	TTC Treatment	Biofilm biomass reduction % Phage Vs PBS	TTC Vs PBS
PA10145FU	1.02	1.46	0.56	30.14	61.64
49669841A	0.03	0.39	0.03	92.27	91.50
63-8199	0.14	1.88	0.27	92.55	85.63
63-7780	0.03	0.11	0.06	72.72	45.45
49669854	0.08	0.71	0.14	88.73	80.28
63-5713	0.17	1.13	0.35	84.96	69.02
63-6299	0.09	0.45	0.14	80	31.11
63-6598	0.078	0.37	0.1047	78.38	72.97
49540983	0.219	0.8	0.26667	72.50	66.25

Table 6: Comparison of effect of phage and tetracycline (TTC) treatment on *S. aureus* biofilm reduction

Isolates ID	OD measurement after: Phage treatment	PBS treatment	TTC Treatment	Biofilm biomass reduction % Phage Vs PBS	TTC Vs PBS
SA6538	0.38	0.84	0.06	54.76	78
49029122	0.04	0.1	0.08	60	20
63-7334	0.08	0.22	0.3	63.63	+36.36
41535062	0.03	0.15	0.04	80	73.33
63-3534	0.0373	0.11	0.053	63.63	54.54
60262911	0.04467	0.15	0.0627	73.33	60
47597332	0.071	0.24	0.044	70.83	83.33

Table 7: Wound size of excisional wound of diabetic mice infected with MDR *S. aureus* isolate SA63-2498 and treated with phage cocktail AB-SA01 or controls.

Treatment group	Mice ID	Day 0	Day 3	Day 5	Day 7	Day 10
Not-infected-phage treated	3-1R	6	5.7	5.35	4.15	1.5
	3-NEM	6	5.8	4.6	4.05	0
	3-RL	6	5.7	5.65	4.4	0
	3-L	6	5.5	5.2	3.95	0
	1-1R	6	5.7	4.9	4.95	0
	1-RL	6	5.45	4.95	3.3	1.2
	2-RL	6	5.5	5	4.45	0
	1-2R	6	5.4	5.6	5.5	0
	Mean ± err	**6 ± 0.0**	**5.59 ± 0.15**	**5.16 ± 0.36**	**4.34 ± 0.66**	**0.34 ± 0.63**
Infected-PBS treated	4-NEM	6	5.85	5.65	6.75	6.2
	4-1L	6	5.8	5.75	6.3	6.1
	4-2R	6	5.6	5.7	6.15	7.15
	5-NEM	6	6	6	6.5	8.6
	5-1L	6	5.9	5.95	6.55	8.95
	5-2R	6	5.8	5.9	6.4	8.75
	10-RL	6	5.75	5.85	6.6	8.5
	Mean ± err	**6 ± 0.0**	**5.81 ± 0.12**	**5.83 ± 0.13**	**6.46 ± 0.2**	**7.75 ± 1.23**
Infected-phage treated	7-1R	6	5.95	5	4.45	0
	7-1L	6	5.95	5.5	4.9	0
	7-RL	6	5.9	4.85	4.6	0
	7-2R	6	6	5.85	4.6	0
	9-NEM	6	5.9	4.85	5.05	1.55
	9-1R	6	5.9	5.7	5.35	0
	9-1L	6	5.85	4.85	4	0
	9-RL	6	6.05	5.65	4.7	0
	Mean ± err	**6 ± 0.0**	**5.94 ± 0.06**	**5.28 ± 0.43**	**4.71 ± 0.41**	**0.19 ± 0.55**
Infected-vancomycin treated	6-1R	6	6.7	6.1	4.45	0
	6-RL	6	7	6	5.05	0
	6-2R	6	6.95	6.45	3.6	0
	8-NEM	6	7	6.65	4.2	0
	8-1L	6	7	6.85	1.8	0
	8-2R	6	7	6.4	4.7	0
	Mean ± err	**6 ± 0.0**	**6.94 ± 0.12**	**6.41 ± 0.32**	**3.97 ± 1.17**	**0 ± 0.0**

Table 8: Bodyweight of excisional wound experiment mice

Treatment group	Mice ID	Day 0	Day 3	Day 5	Day 7	Day 10
Not-infected-phage treated	1-1R	19.9	19.9	19.5	19.6	19.6
	1-RL	19.3	19.3	19.3	19.2	19.6
	1-2R	17.2	17.3	17	16.2	16.3
	2-RL	18.6	18	18	18.3	18.1
	3-NEM	21	20.9	20.4	20.3	19.9
	3-1R	20.4	20.3	19.9	20	19
	3-L	19.5	19.7	19.6	19.4	19.4
	3-RL	17.6	17.9	17.5	16.8	16.2
	Mean + err	**19.19 ± 1.32**	**19.16 ± 1.29**	**18.9 ± 1.23**	**18.73 ± 1.5**	**18.52 ± 1.5**
Infected-PBS treated	4-NEM	20.6	19.7	18.8	19.2	18.4
	4-1L	18.5	17.3	16.1	16	16.5
	4-2R	20.1	18.9	17.3	18	18.4
	5-NEM	19	16.9	16.2	16.1	15.5
	5-1L	20.3	19.7	18.2	17.5	17.6
	5-2R	18.7	17	15.4	15.1	N/A
	10-RL	16.5	16.3	16.3	15.1	15.3
	Mean + err	**19.1 ± 1.41**	**17.97 ± 1.42**	**16.9 ± 1.24**	**16.71 ± 1.56**	**16.95 ± 1.39**
Infected-phage treated	7-1R	20.6	17.7	17.6	17.5	18.4
	7-1L	22.4	19.7	19.3	19.8	19.3
	7-RL	19.3	17.7	17.2	17.4	17.3
	7-2R	22	20.3	19.2	18.2	17.9
	9-NEM	18.9	16.8	16.3	15.9	16.7
	9-1R	22.2	19.8	19.6	18.9	20
	9-1L	22	21	20.3	19.9	20.4
	9-RL	21	20.9	19.2	19.3	19.8
	Mean + err	**21.05 ± 1.35**	**19.24 ± 1.61**	**18.59 ± 1.38**	**18.36 ± 1.38**	**18.73 ± 1.35**
Infected-vancomycin treated	6-1R	21.6	19.7	18.2	17.7	18.7
	6-RL	20	18.2	17.2	17.6	17.5
	6-2R	21	20.6	19.3	18.4	18.7
	8-NEM	18.9	17.6	16.6	16.2	16.9
	8-1L	19.8	19.1	17.3	17.9	17.5
	8-2R	18.7	16.4	16.4	16.7	16.7
	Mean + err	**20.0 ± 1.14**	**18.6 ± 1.51**	**17.5 ± 1.08**	**17.42 ± 0.81**	**17.67 ± 0.86**

Table 9: Fasting blood glucose level (BGL, in mmol/L) of mice from a week of post-STZ injection

	Dates and BGL				
Mice ID	7-8/8/18	9-10/8/18	13-14/8/18	20-21/8/18	27-28/8/18
1-NEM	7.5	6.2	8.8	8.7	8.9
1-R	9.8	8.9	17.8	18.9	14.7
1-L	8.4	8.4	11.5	11	12.5
1-RL	7.4	6.8	12.4	14.3	9.8
1-2R	14.2	18.5	18.5	24	26.1
2-NEM	18.2	20.6	25	19.6	25.4
2-R	9.2	8.8	16.6	24.1	14.1
2-L	8.2	11.5	Died	----	----
2-RL	9.2	20.6	21	21.4	23.8
2-2R	14	21.5	21	21.9	26.6
3-NEM	7	9.5	21.6	22.6	20.5
3-R	7.3	13.3	18	22.1	17.5
3-L	10.5	12.5	18	22	19.3
3-RL	8.4	15.3	19.9	21.5	22.1
3-2R	5.8	6.6	8.8	6.8	7
4-NEM	11.3	15.4	19.9	19.8	20
4-R	8.5	11.6	Died	----	----
4-L	10.4	15.1	22	22.3	20.4
4-RL	7.2	7.2	11.5	11.8	Died
4-2R	12.5	17.2	17.6	25.7	18.9
5-NEM	12.4	13.4	20.3	20.9	19.5
5-2R	10.9	13.8	11.6	14.1	8.2
5-L	8	8.4	18	17.8	14.9
5-RL	10.9	12	18.6	20.9	19.4

5-2R	7	13.3	20.8	23.5	19.6
6-NEM	17.8	14.7	11.1	11.1	10.8
6-R	9.6	18.1	21.9	26.4	19.3
6-L	19.5	22.8	25.3	24.6	23.9
6-RL	18.1	20.2	11.1	19	24.1
6-2R	14.8	23.6	24.2	22.1	21.9
7-NEM	12.8	16.2	18	17.1	17.3
7-R	10.2	16	20	18.7	18.4
7-L	12.1	18.1	21.9	21.4	24.1
7-RL	12.7	19.5	19.1	20.6	20.9
7-2R	12.3	16.9	23	22.9	18
8-NEM	12.2	28.1	19.5	21	21.7
8-R	11.2	17	16.1	16.8	16.4
8-L	9.1	14	18.6	22.6	18.6
8-RL	7	8.2	7.2	7.6	7.9
8-2R	12.1	17.8	18.9	20.4	19.9
9-NEM	22.1	18	17.9	24.6	23.3
9-R	14.2	14.2	14	16.1	11.9
9-L	12.1	15.3	14.8	11.9	7.4
9-RL	9.8	16.5	21.5	17	11
10-NEM	12.8	13.6	22.6	26.6	23.9
10-R	Died	----	----	----	----
10-L	10.1	24.4	31.4	31.9	Hi
10-RL	13.2	15.1	22.4	Hi	27.5

Table 10: Efficacy of disinfectants and tetracycline on *S. aureus* biofilms.

	Mean OD after treatment by:			% MBR because of:	
Bacterial ID	PBS	AB-SA01	2.5% CPC	AB-SA01	2.5% CPC
63-5897	0.6	0.3	0.8	-50.0	+33.3
48561341	0.5	0.3	0.7	-40.0	+40.0
63-6378	0.2	0.1	0.3	-50.0	+50.0
	PBS	AB-SA01	Triton x-100	AB-SA01	Triton x-100
48556348	0.1	0.04	0.4	-60.0	+30.0
63-6758	0.4	0.1	0.6	-75.0	-40.0
63-2498	0.2	0.04	0.7	-80.0	+250.0
	PBS	AB-SA01	Tetracycline	AB-SA01	Tetracycline
63-3534	0.1	0.04	0.05	-60.0	-50.0
60262911	0.2	0.04	0.05	-80.0	-75.0
41535062	0.2	0.05	0.06	-75.0	-70.0

Key: - and + indicate the biofilm biomass decrease and increase, respectively, because of the treatment compared to the PBS treatment.

Table 3: Phage cocktail and component phages susceptibility and biofilm reduction (in percentage - %) after phage cocktail treatment of the selected bacterial isolates

		Susceptibility to phages									% biofilm reduction	
No.	Bacterial isolate	J-Sa-36	Sa-83	Sa-87	AB-SA01	Pa-93	Pa-204	Pa-222	Pa-223	AB-PA01	AB-SA01	AB-PA01
1.	*S. aureus* KUB7	S	S	S	S	n/a	n/a	n/a	n/a	n/a	72.84	n/a
2.	63-6538	S	S	S	S	n/a	n/a	n/a	n/a	n/a	87.66	n/a
3.	63-2498	S	S	S	S	n/a	n/a	n/a	n/a	n/a	80.00	n/a
4.	63-6565	S	S	S	S	n/a	n/a	n/a	n/a	n/a	75.68	n/a
5.	GFP PAO1	n/a	n/a	n/a	n/a	S	S	S	S	S	n/a	73.36
6.	63-6598	n/a	n/a	n/a	n/a	S	I	S	S	S	n/a	88.24
7.	63-5497	n/a	n/a	n/a	n/a	S	S	S	S	S	n/a	80.68
8.	63-6036	n/a	n/a	n/a	n/a	I	S	S	S	S	n/a	72.78

Key: S = Susceptible, I = Intermediate, n/a = Not applicable

Table 12: Source and antimicrobial susceptibility of *S. aureus*

				Antimicrobial Susceptibility Test Result of Clinical and Lab Strains of S. aureus																		
ID	Date collected	Site	Cefoxitin	Benyzlepeni	Oxacillin	Gentamy	Ciproflox	Induced Clindam	Erythromyci	Clindamycin	Linezolid	Daptomycin	Teicoplanin	Vancomyc	Tetracyclir	Nitrofurant	Fusidic Aci	Mupirocin	Rifampicin	TSM	MRSA	MDR
SA4220	….....	Lab strain	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
SA6538	….....	Lab strain	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	….....	S	S	N	N
48556348	7/12/2016	RAH	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	….....	S	S	N	N
63-7148	21/03/2017	QEH	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	….....	S	S	N	N
63-169	8/01/2017	RAH	P	R	R	S	R	N	R	R	S	S	S	S	R	S	S	….....	S	S	Y	Y
63-2498	1/02/2017	GP	P	R	R	S	R	P	R	R	S	S	S	S	S	S	S	….....	S	S	Y	Y
63-7647	21/03/2017	RAH	P	R	R	S	S	P	R	R	S	S	S	S	S	S	S	….....	S	S	Y	Y
63-5897	5/03/2017	GP	N	R	S	S	S	N	R	R	S	S	S	S	S	S	S	S	S	S	N	N
63-6758	14/03/2017	GP	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
60271003	17/02/2017	FMC	P	R	R	S	S	N	S	I	S	S	S	S	S	S	S	S	S	S	Y	Y
63-6354	7/03/2017	GP	P	R	R	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	Y	Y
63-6126	5/03/2017	LMH	N	R	R	S	R	N	R	R	R	R	R	I	S	S	S	S	S	S	Y	Y
S04-5089	14/03/2017	LMH	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
04-5755S	18/04/2017	QEH	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
62-9282	8/01/2017	RAH	P	R	R	S	S	N	R	R	S	S	S	S	S	S	S	S	S	R	Y	Y
63-2749	27/02/2017	RAH	P	R	R	S	R	N	S	S	S	S	S	S	S	S	S	S	S	S	Y	Y
63-5612	27/02/2017	LMH	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-5273	27/02/2017	Country	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-7019	14/03/2017	RAH	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
46834854	7/12/2016	RAH	P	R	R	S	S	P	R	R	S	S	S	S	S	S	S	S	S	S	N	Y
63-6124	11/12/2016	RAH	N	R	S	S	S	N	R	R	S	S	S	S	S	S	S	S	S	S	N	Y
48392361	7/12/2016	GP	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-5853	5/03/2017	LMH	N	R	S	S	S	N	S	S	S	R	R	R	S	S	S	S	S	S	N	Y
63-8600A	8/01/2017	GP	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
90271071	7/12/2016	RAH	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-8782S	30/03/2017	LMH	P	R	R	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	Y	Y
63-5339	27/02/2017	Country	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-5402	27/02/2017	GP	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-7252	21/03/2017	GP	N	R	S	S	R	N	R	S	S	S	S	S	S	S	S	S	S	S	N	Y
63-3395	9/02/2017	GP	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
62-9187	8/01/2017	GP	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-2548	1/02/2017	RAH	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
99279922	7/12/2016	LMH	N	R	S	S	S	P	R	R	S	S	S	S	S	S	S	S	S	S	N	Y
47597332	30/11/2016	FMC	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-3534	5/02/2017	RAH	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-2482	1/02/2017	Country	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-145	8/01/2017	GP	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
S04-5216	21/03/2017	RAH	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
60262911	12/02/2017	FMC	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-6832	14/03/2017	MOD	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
62-8701	8/01/2017	LMH	N	R	S	S	S	N	S	S	S	R	R	R	S	S	S	S	S	S	N	Y
93952969	12/02/2017	FMC	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-2599	1/02/2017	GP	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-5656	5/03/2017	GP	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-6352	7/03/2017	RAH	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-3485	4/02/2017	GP	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
48561341	7/12/2016	GP	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
49639617	30/11/2016	FMC	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-5222	27/02/2017	QEH	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-7334	21/03/2017	GP	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
41535062	30/11/2016	FMC	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-6036	5/03/2017	LMH	N	R	S	S	S	N	R	R	S	S	S	S	S	S	S	S	S	S	N	N
49029122	7/12/2016	RAH	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-6378	7/03/2017	GP	N	R	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N
63-5289	27/02/2017	GP	N	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	N	N

Table 12: Source and antimicrobial susceptibility of *P. aeruginosa*

			Antimicrobial Susceptibility Test Result of Clinical and Lab Strains of P. aeruginosa									
ID	**Date collected**	**Site**	**Ticarcillin/Clavu**	**Piperacillin/Tazol**	**Ceftazidime**	**Cefepime**	**Meropenem**	**Amikacin**	**Gentamyci**	**Tobramycin**	**Ciprofloxa**	**Norfloxacin**
PAO1 GFP	….....	Lab strain	S	S	S	S	S	S	R	S	S	S
PA15692	….....	Lab strain	R	R	S	S	S	S	S	S	S	S
PAO10145 FU	….....	Lab strain	R	S	S	S	S	S	S	S	S	S
62-8001	11/12/2016	QEH	S	S	S	S	I	S	S	S	R	I
63-5405	27/02/2017	Modbury	R	R	S	S	S	S	S	S	S	S
63-6598	9/03/2017	QEH	S	S	S	S	S	S	S	S	S	S
63-7648	21/03/2017	RAH	R	S	S	S	S	S	S	S	S	S
63-7786	21/03/2017	RAH	S	S	S	S	S	S	S	S	S	S
63-7996	28/03/2017	RAH	S	S	S	S	S	S	S	S	R	S
63-9029	4/03/2017	GP	S	S	S	S	S	S	S	S	S	S
63-9513	21/03/2017	RAH	S	S	S	S	S	S	S	S	S	S
04-5755P	18/04/2017	QEH	S	S	S	S	S	S	S	S	S	S
62-8028A	11/12/2016	QEH	R	S	S	S	S	S	S	S	S	S
62-8241A	11/12/2016	RAH	S	S	S	S	S	S	S	S	S	S
63-8882P	30/03/2017	GP	S	S	S	S	S	S	S	S	S	S
63-7651	28/03/2017	QEH	R	R	R	R	S	S	S	S	S	S
63-8795	30/03/2017	RAH	R	R	R	R	S	S	S	S	S	S
63-8782P	30/03/2017	LMH	S	S	S	S	S	S	S	S	S	S
63-296P	18/04/2017	RAH	R	S	S	S	S	S	S	S	S	S
49669841A	9/02/2017	FMC	R	S	S	S	S	S	S	S	S	S
48489280B	7/12/2016	RAH	R	S	S	S	S	S	S	S	S	S
62-2220	1/02/2017	GP	S	S	S	S	S	S	S	S	S	S
63-5713	7/03/2017	LMH	S	S	S	S	S	S	S	S	S	S
63-6728	14/03/2017	RAH	R	S	S	S	S	S	S	S	S	S
63-7889	28/03/2017	RAH	R	S	S	S	S	S	S	S	S	S
63-3521	9/02/2017	LMH	R	S	S	S	S	S	S	S	S	S
63-5497	27/02/2017	RAH	S	S	S	S	S	S	S	S	S	S
63-6299	7/03/2017	RAH	S	S	S	S	S	S	S	S	S	S
63-3624	6/02/2017	GP	S	S	S	S	S	S	S	S	S	S
63-6608	14/03/2017	RAH	R	S	S	S	S	S	S	S	S	S
63-7780	21/03/2017	RAH	R	S	S	S	I	I	S	S	R	R
63-7871	28/03/2017	GP	S	S	S	S	S	S	S	S	S	S
63-8199	28/03/2017	LMH	S	S	S	S	S	S	S	S	S	S
63-8601	28/03/2017	GP	S	S	S	S	S	S	S	S	S	S
63-8788	30/03/2017	LMH	R	S	S	S	S	S	S	S	S	S
49540983	7/12/2016	RAH	R	R	R	R	S	S	S	S	S	S
49669854	17/02/2017	FMC	R	S	S	S	S	S	S	S	S	S
P04-5393	30/03/2017	RAH	R	R	R	R	S	S	S	S	R	I
63-5656	5/03/2017	GP	S	S	S	S	I	S	S	S	S	S

Key: RAH – Royal Adelaide Hospital, QEH – Queen Elizabeth Hospital , FMC – Flinders Medical Centre, GP , LMH , S – susceptible, I – intermediate, R – resistant, N -negative/not, Y- yes, MRSA – methicillin resistant S. aureus , MDR - multidrug resistant

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