Modulating the Susceptibilities of Bacteria to Antibiotics using Manuka Honey

A thesis submitted to the University of Manchester for the degree of

Doctor of Philosophy

In the Faculty of Biology, Medicine and Health

(2021)

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Word counts: 39.879

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List of Abbreviations

AgNP	Silver nanoparticles
a$_w$	Water activity
BLAST	Basic Alignment Search Tool
BMI	Body Mass Index
BNF	British National Formulary
CFU	Colony Forming Units
DHA	Dihydroxyacetone
DFU	Diabetic foot ulcer
DM	Diabetes mellitus
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
EUCAST	European Committee on Antimicrobial Susceptability Testing
LPS	Lipopolysaccharide
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MGO	Methylglyoxal
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRDs	Moisture-retentive dressings
MRJP	Major Royal Jelly Protein
NGS	Next Generation Sequencing
NHS	National Health Service
NICE	National Institute for Health and Care Excellence

NPA	Non-peroxide activity
OD	Optic density
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PUs	Pressure ulcers
QS	Quorum sensing
RCTs	Randomised control trials
SCV	Small Colony Variants
UMF	Unique Manuka Factor
UV	Ultraviolet

Abstract

Background. The clinical application of Manuka honey, particularly for the treatment of chronic wound infections, has recently gained momentum. With the widespread use of honey-impregnated wound dressings, however, concerns have been raised regarding the potential for prolonged honey exposure to drive changes in bacterial antimicrobial sensitivity and virulence. This doctoral thesis aims to evaluate the bacteriological effects of bacterial passaging in planktonic and biofilm growth modes in the presence of a commercially available Manuka honey wound gel. **Methods.** Eight bacteria, including chronic wound isolates, were repeatedly exposed to sub-therapeutic concentrations of Manuka honey over ten passages (P10) and again following ten additional passages in honey-free media (X10) using an agar-based diffusion system. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum biofilm eradication concentration (MBEC) of honey and antibiotic susceptibilities were determined before and after long-term exposure to Manuka honey. Biofilm passaging was achieved using an MBEC device comprising sub-therapeutic concentrations of wound gel prepared in Mueller Hinton media. Passaged bacteria were subjected to further bacteriological analysis. Biofilm-formation was quantified using a crystal violet assay, and bacterial pathogenicity was assessed via a *Galleria mellonella* waxworm model. Passaged planktonic isolates exhibiting significant changes in virulence were further investigated for changes (versus parent strain) in haemolysin, coagulase, DNase, pyocyanin and motility. Where significant changes in virulence or antimicrobial sensitivity profiles were observed, passaging experiments were repeated through the repeated exposure of biofilm growth modes to subtherapeutic concentrations of honey wound gel using an MBEC assay[TM.] Here, changes in colony morphology, antimicrobial susceptibilities, biofilm-formation ability, dynamic growth rate and exotoxin production were assessed in biofilm derived isolates and compared to passage controls. Phenotypic analyses were conducted in conjunction with whole-genome sequencing of differential colony morphotypes **Results.** Compared to parent strains, moderate changes ($\leq$ 1-fold) in susceptibility to Manuka honey wound gel were observed following passaging. Staphylococcal strains exhibited a $\geq$4-fold increase in susceptibility to vancomycin. Additionally, transient phenotypic resistance to erythromycin following exposure to Manuka honey was noted in *S. epidermidis*. Increased MBECs for gentamicin were documented in both strains of *P. aeruginosa*, with strain WIBG 2.2 representing a 7-fold reduction in susceptibility. Relative-pathogenicity significantly increased after honey exposure in 4/8 bacterial strains, including *Staphylococcus aureus* WIBG 1.2, *S. epidermidis*, *P. aeruginosa* WIBG 1.3 and *Escherichia coli*. The enhanced virulence in pseudomonads and *S. epidermidis* occurred in association with enhanced biofilm formation and haemolysis, in addition to increased pyocyanin, swimming and swarming motility in *P. aeruginosa.* Following passaging with Manuka honey, one strain of *S. aureus* displayed non-pigmented colonies with reduced virulence, haemolysin, DNase, and coagulase. When passaged in sessile form, both *P. aeruginosa* and *S. epidermidis* exhibited colonies with reduced diameter and reduced sensitivity to gentamicin and vancomycin, respectively. Genome analysis of *P. aeruginosa* variants identified point mutations in *fbcH*, *cheB*, *mcpB*, *hudA* and *lasR* genes, while point mutations in *cdaR*, *sdrG*, *scrK* and *lipA* genes were observed in biofilm-derived colony variants of *S. epidermidis* when compared to passage controls. Enhanced virulence *in vivo* was observed in both *P. aeruginosa* and *S. epidermidis* biofilm variants in conjunction with increased biofilm formation, whilst *P. aeruginosa* also demonstrated overproduction of extracellular protease, elastase and pyocyanin. **Conclusion.** These data suggest that repeated exposure of wound isolates to Manuka honey wound gel led to variable changes in antimicrobial susceptibility, biofilm formation, and relative-pathogenicity. *S. aureus* 1.6 showed reduced virulence, biofilm formation, haemolysin, Dnase and coagulase while both *P. aeruginosa* and *S. epidermidis* exhibited enhanced virulence and biofilm formation. Enhanced virulence and biofilm formation in adapted *P. aeruginosa* and *S. epidermidis* could potentially impact wound healing, but all isolates remained sensitive to in-use concentrations of Manuka honey.

Declaration

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Research Contributions

First author papers

Mokhtar JA, McBain AJ, Ledder RG, Binsuwaidan R, Rimmer V and Humphreys GJ (2020) Exposure to a Manuka Honey Wound Gel Is Associated with Changes in Bacterial Virulence and Antimicrobial Susceptibility. Front. Microbiol. 11:2036. doi: 10.3389/fmicb.2020.02036

Pre-submission papers

Mokhtar JA, McBain AJ, Ledder RG and Humphreys GJ. Adaptation of Biofilm Derived Bacteria Following Exposure to a Manuka Honey Wound Gel.

Poster presentations

ASM Microbe Meeting, Atlanta, USA **(2018)**

J.A. Mokhtar, A.J. McBain, R.G. Ledder, G.J. Humphreys

Exposure to Manuka Honey Modulates Antibiotic Susceptibility in Wound Isolates.

The 7th European Clinical Microbiology Congress, London, UK **(2018)**

J.A. Mokhtar, A.J. McBain, R.G. Ledder, G.J. Humphreys

Exposure to Manuka Honey Modulates Antibiotic Susceptibility in Wound Isolates.

Acknowledgements

First, I would like to express my thanks to Allah for his help and support. I would also like to express my deepest appreciation to my main supervisor Dr. Gavin Humphreys for his continuous guidance, support, and motivation. Without his valuable guidance, this project would not have been completed.

Special thanks also go to my co-supervisors Prof. Andrew McBain and Dr. Ruth Ledder for their advice and guidance, especially during the lab work and writing the manuscripts and thesis.

I would also like to thank all the members of the Microbiology Research Group and my friends in the microbiology lab, Reem, Hana, Muna, Sajwa, Sarah, Mona, Maha,Victoria and Ahmad for their support and the enjoyable time we have had during the PhD journey. Special thanks go to my best friends, Hind and Rania, for their help in taking care of my kids and encouraging me during my difficult times to continue this journey as well as for the most enjoyable time we have had together.

I would like to gratefully thank my parents and dedicate this thesis to them. Thank you for your support, praying for me and encouragement, there are no words expressing my gratitude and appreciation to you. Also, special thanks to my brothers and sisters for being supportive during the project.

My lovely small family, I would like to express my great thanks for being supportive, your patience for me at my busy time, and for showing faith in me. Finally, I would like to acknowledge my sponsor, King Abdulaziz University, for funding my PhD.

Chapter 1

General Introduction

1.1 Overview

Chronic wounds are a major economic burden on the health system, contribute to substantial declines in patient quality of life, and may be associated with severe outcomes, such as limb amputation or early death (Järbrink et al., 2017). Age (> 60 years), obesity (BMI ≥ 30) and comorbidities, such as diabetes and venous insufficiency, contribute to the increase in the number of patients with chronic wounds (Martin, 2001, Sen, 2019). In the UK, about 2.2 million patients have a wound that affects their quality of life leading to increased healthcare costs (Guest et al., 2015, Guest et al., 2017). Most chronic wounds are colonized by multiple microorganisms, however, infection occurs when the balance between the host immune status and the number of bacteria is disturbed (Wysocki, 2002, Salcido, 2007). As a result of this, delayed wound healing may occur and aggressive treatment is usually required (Siddiqui and Bernstein, 2010).

Given the recent surge in antibiotic-resistant bacteria, new treatment methods have been called for (Cheesman et al., 2017). In recent years, natural products such as honey, have attracted increased research attention as potential topical alternatives to antibiotics (Allen et al., 2014b, Cooper, 2014, Eteraf-Oskouei and Najafi, 2013). In several countries, medical honey is certified for use in wound dressings and are formulated into ointments and gels (Carter et al., 2016b). Manuka honey is the most common medicinal honey used at present (Cooper, 2016). This honey is extracted from the Manuka tree, *Leptospermum scoparium*, which grows in New Zealand and Eastern Australia. It has attracted a great deal of attention from researchers for its biological properties, particularly its antimicrobial and antioxidant capability (Alvarez-Suarez et al., 2014b, Adams et al., 2009, Atrott and Henle, 2009, Kato et al., 2012, Johnston et al., 2018). Many studies reported that Manuka honey has *in vitro* activity against planktonic bacteria as well as anti-biofilm activity (Roberts et al., 2012a, Maddocks et al., 2012, Maddocks et al., 2013).

The application of antimicrobial wound dressings for both the prevention and treatment of chronic wounds is prevalent within healthcare systems (Molan and Rhodes, 2015). Antimicrobial wound dressing expenses in the UK rose by £ 28 million between 1997 and 2016 (Hussey et al., 2019). Manuka honey containing advanced dressings typically exert a broad spectrum of activity and act at multiple sites within the bacterial cell (V Ranall et al., 2012, Bradshaw, 2011). Maintaining high amounts of antimicrobials in the wound area is, however, required to reduce viable bacteria before resistance strains develop. Here, dilution effects have been reported, for example, as a result of wound fluid (Bang et al., 2003). Excessive and prolonged use of antimicrobial dressings has also led to increased concern about the emergence of bacterial resistance and the potential consequences on virulence and pathogenicity (Blair et al., 2009, Cooper et al., 2010b). Similar to antibiotics, the widespread use of honey may well provide a selective pressure that is responsible for the development of honey-resistant strains.

1.2 Chronic wounds

Chronic wounds are those that do not heal in an orderly manner and tend to remain in one stage of wound healing, mainly the inflammatory phase, for extended periods (Harding et al., 2002). Wound healing is a regulated and complex process that is important in the maintenance of the skin barrier functions and it requires the intricate interplay of various factors. Therefore, the stagnation of wounds in one healing stage due to unbalanced tissue loss and degradation of molecules, such as collagen, results in the development of a chronic wound (Han and Ceilley, 2017). Chronic wounds have a substantial economic burden to the healthcare system, and they may result in reduced quality of life of the patient (Siddiqui and Bernstein, 2010). Chronic wound patients often also suffer from long-term pain, social

isolation, poor sleep, limitation in mobility, and sometimes require limb amputation (Herber et al., 2007, Margolis et al., 2002).

1.2.1 Wound bioburden and infection

Intact skin is necessary to control the microbial population on the skin surface itself. Once injury has occurred, the skin integrity is lost and the subcutaneous tissue becomes exposed, providing an environment for microbial colonization (Siddiqui and Bernstein, 2010, Howell-Jones et al., 2005b). However, the infection occurs only when the host defence mechanisms cannot control the bacterial bioburden, and the virulence factors produced by the microorganisms cause further tissue damage (Edwards and Harding, 2004). It has been claimed that wound infection can happen when more than 10^5 microbial cells per gram of tissue exist in a wound (Mustoe et al., 2006, Robson, 1997). Infection delays wound healing and displays active clinical features of infections due to the release of toxins (Edwards and Harding, 2004).

Multiple virulence factors help bacteria to invade the wound tissue, cause disease and escape from the host defences (Cross, 2008). The attachment of bacterial cells either to the surface of the target cell or to components of the extracellular matrix is considered the first step in the initiation of infection (Cooper, 2002). Several bacterial structures facilitate attachment, such as fimbriae, capsules and cell-wall binding proteins (Cooper, 2002). In addition, bacteria can invade deeper tissues by producing different enzymes that breakdown the wound's extracellular matrix. For instance, staphylococci produce hyaluronidase to break down peptidoglycan in connective tissue, staphylokinase to dissolve fibrin clots, and lipase and nuclease to collapse lipid and nucleic acid, respectively (Cooper, 2002).

1.2.2 Common bacterial species present in chronic wounds

Many chronic wound infections are polymicrobial and are usually contaminated by microorganisms from the environment, surrounding skin, and endogenous origins (Siddiqui and Bernstein, 2010, Bowler et al., 2001). Widespread opinion among wound care specialists is that facultative or aerobic pathogens such as *Staphylococcus aureus* (93.5% of the ulcers) and *Pseudomonas aeruginosa* (52.2%) are the primary reasons for infection and delayed healing in both chronic and acute wounds (Leaper, 1998, Gjødsbøl et al., 2006). Other organisms causing chronic wound infections include *Enterococcus faecalis* (71.1%), *Proteus* spp. (41.3%), beta-hemolytic streptococci (24.7%), anaerobes such as *Bacteroides* spp. (39.1%) and fungi (Gjødsbøl et al., 2006, Siddiqui and Bernstein, 2010). By using molecular techniques, it has been confirmed that the greatest number of wounds are polymicrobial and the majority of microbes are aerobic (Frank et al., 2009, Davies et al., 2001). Such an outlook has been made based on studies performed largely during the previous two decades (Davies et al., 2001, Dowd et al., 2008, Rhoads et al., 2012, Oates et al., 2012a).

Chronic wounds have been shown to be colonized and/or infected by different antibiotic-resistant organisms (Howell-Jones et al., 2005a). For instance, a cohort study was performed to estimate the risk of methicillin-resistant *Staphylococcus aureus* (MRSA) in chronic wounds patients. They found that 30% (166/545) of these patients with chronic ulcers were colonized with MRSA (Roghmann et al., 2001). In addition, most of the infected leg ulcers caused by *P. aeruginosa* isolates found to be resistant to ciprofloxacin (Colsky et al., 1998, Serra et al., 2015). Consequently, the management of chronic wounds becomes more challenging and developing new therapeutic approaches are needed (Howell-Jones et al., 2005a).

1.2.3 Common types of chronic wounds

1.2.3.1 Diabetic ulcers

Diabetes mellitus (DM) has increased dramatically over the past decade and it is one of the major comorbidities in healthcare systems (Gianino et al., 2018). Diabetic foot ulcer (DFU) is a common complication of DM and affects about 15% of these patients during their lifetime (Yazdanpanah et al., 2018). DFU is considered as a main cause of morbidity in diabetic patients and it can lead to infection, gangrene, amputation, and even death if not treated (Snyder and Hanft, 2009). The aetiology of the diabetic foot ulcer is peripheral neuropathy and ischemia that may lead to ulceration and deformity of the tissues as well as a build-up of fluids. Ulceration leads to insufficient oxygenation of tissues that lead to the formation of a chronic wound (Boulton et al., 2005, Yazdanpanah et al., 2018).

Diabetic ulcers mostly experience infection with Gram-positive bacteria, (*Staphylococcus aureus* and *Enterococcus*), and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Klebsiella* spp., *Escherichia coli* and anaerobes) (Shankar et al., 2005). To identify bacterial diversity in DFU, Dowd et al. (2008) evaluated 40 tissue samples from 40 diabetic patients with ulcers using a combination of pyrosequencing, shotgun Sanger sequencing, and denaturing gradient gel electrophoresis. They reported that all ulcers were poly-microbial and the most commonly isolated bacteria were corynebacteria (75%), staphylococci (32%), streptococci (52%), enterococci (25%) and *Pseudomonas* spp. (20%). Additionally, they found multiple different anaerobic bacteria such as *Bacteroides*, *Finegoldia* and *Anaerococcus* spp (Dowd et al., 2008).

1.2.3.2 Pressure ulcers

Pressure ulcers (PUs) are a serious medical problem that frequently occur in individuals who have reduced movement of body parts, such as patients in intensive care units or those with paralysis (Agale, 2013). Pressure sores usually occur when the pressure on the tissues is

high, mainly over the bony prominences, which restrict the blood flow to the tissue resulting in tissue ischemia and death. The estimated cost of pressure ulceration to the NHS ranges from £507 to £530.7 million a year (Guest et al., 2017). Infection is a frequent problem in PUs and increases the risk of morbidity and mortality for patients (Ebright, 2005). The most common micro-organisms causing infection in PUs are *S. aureus* and Gram-negative bacilli such as *P. aeruginosa*, *Enterobacteriaceae* and *Acinetobacter baumannii* (Landis, 2008). In addition, multi-drug resistant organisms like methicillin-resistant *S. aureus* (MRSA) are frequently isolated from patients with pressure ulcer infection, which can lead to poor prognostic factors and death (Ellis et al., 2003).

1.2.3.3 Venous ulcers

Chronic venous insufficiency and chronic venous ulceration are common chronic diseases and they account for more than 5% of the world population suffers from venous disease (Ruckley, 1997). They develop from the damage of veins due to lack of blood flow to the heart and backward flow of the blood, resulting in ischemia and reperfusion injury that cause tissue damage and subsequent wounds (Etufugh and Phillips, 2007). The valvular incompetence leads to the distention and stretching of the blood vessels to accommodate the excess blood, resulting in venous hypertension (Falanga and Eaglstein, 1993, Etufugh and Phillips, 2007). Vascular hypertension leads to the leakage of blood and other components from the stretched vessels into the surrounding tissues, resulting in the deposition of pigments in the gaiter area of the leg (Falanga and Eaglstein, 1993). The ulcers are located in the gaiter area between the ankle and the calf and are recurrent and can persist for several years. Patients may develop complications such as cellulitis, malignant transformation and osteomyelitis from the persistent ulcers (Etufugh and Phillips, 2007).

The most common predisposing factors are obesity, old age, deep vein thrombosis, congestive heart failure and trauma or surgery to the leg (Scott et al., 1995, Abbade and Lastória, 2005). Clinical infection of venous ulcers is linked with wound breakdown and impaired healing (Zmuzdinska et al., 2005). Various bacterial species have been recognised in venous leg ulcers through routine bacteriological culture as well as molecular techniques, including *S. aureus*, *P. aeruginosa*, *Corynebacterium* spp., *Finegoldia* spp., *Proteus mirabilis*, and anaerobes (Davis et al., 2008, Wolcott et al., 2009, Moore et al., 2010).

1.3 Management of chronic wounds

Wound care has become one of the most important aspects in the medical field, because the prevalence of chronic wounds and morbidity associated with them has increased worldwide (Han and Ceilley, 2017). The main concepts in wound care are bed preparation by debridement, maintaining moisture balance and choosing an appropriate dressing (Harding et al., 2002, Han and Ceilley, 2017). Debridement refers to the process through which non-viable tissue from a wound gets removed and this can be achieved through surgical or autolytic/enzymatic mechanisms (Atkin, 2016, Han and Ceilley, 2017). The major goal of the debridement is preserving the healthy tissue while ridding necrotic debris which serves as a source for infection and impaired wound healing (Werdin et al., 2009, Han and Ceilley, 2017).

Many wound dressings have been developed to maintain a moist wound atmosphere, facilitate autolytic debridement, and promote granulation and epithelization (Falanga, 2004). There are a broad variety of moisture-retentive dressings (MRDs) that have moisture vapour transmission rates of <35 g/m^2/hr to permit for moist wound healing (Powers et al., 2016). The benefits of MRDs for acute and chronic wounds have been confirmed in clinical trials (Nemeth et al., 1991, Chaby et al., 2007). A prospective randomized trial investigating the management

of venous leg ulcers suggested that the initial healing rates with these dressing plus compression is faster than compression alone (Cordts et al., 1992). Films, foams, hydrocolloids, alginate, and hydrogels are the five basic categories of MRDs (Table 1.1). Films are transparent, thin, and elastic sheets of polyurethane which are the choice dressing for acute surgical wounds and donor sites of split-thickness skin grafts. Foams are bilaminate hydrophobic polyurethane sheets with hydrophilic surface to avoid leakage and microbial contamination and are suitable for mild/moderate exudative wounds (Powers et al., 2016). Hydrocolloid and hydrogels are composed of hydrophilic materials that allow them to maintain a moist environment even with their ability to absorb a certain amount of exudate. Hydrogels may also be used in dry wounds to help promote moisture (Han and Ceilley, 2017). Lastly, alginate dressings which are composed of cellulose-like polysaccharide derived from algae or kelp are ideal for heavily exudative wounds as they are highly absorbent dressings; therefore adverse effects can be observable in dry wounds covered with alginate (Barnett and Varley, 1987).

The recent expansion in wound dressings has focused on combining traditional wound dressings such as foams or hydrogels with antimicrobial compounds such as silver, polyhexamethylene biguanide or honey (Chaby et al., 2007, Bradshaw, 2011). However, these types of dressings may only be appropriate in chronic leg ulcers where infection can be a problem, particularly where biofilm formation may occur (Percival et al., 2008).

Table 1. 1 Moisture-retentive dressings. Adapted from (Wiegand et al., 2015).

Dressing type	Description	Advantages	Disadvantages	Brand-name
Hydrocolloid	Malleable sheets composed of waterproof gels or foams within polyurethane films; suitable to mildly exudative wounds	Simple to apply, waterproof and stimulate granulation tissue.	Drainage, gel formation and not suitable for cavities	Duoderm, NuDerm, Comfeel, Hydrocol, Cutinova and Tegasorb
Alginates	Comprised of polysaccharides derived from kelp and algae, ideal for wounds with high exudate.	Highly absorbent, haemostatic benefits	Not suitable for dry wounds (painful with removal), required frequent dressings changes.	Algiderm, Algisorb, Algosteril, Kaltostat, Curasorb, Melgisorb, SeaSorb,
Hydrogels	Cross-linked hydrophilic polymer, composed of 96% water, Good for dry, necrotic wounds.	Comfortable for the patient and promoting autolytic debridement.	Skin maceration if wound is highly exudative	Vigilon, Nu-gel, Tegagel. FlexiGel, Curagel, Clearsite, Curafil, Curasol, Elasto-Gel, Hypergel, Normgel. And Transigel
Films	Thin layers of elastic polyurethane; used for split-thickness donor skin grafts	Permeable to gas, allows for visualization of the wound, and protect against bacteria.	Poor drainage of fluid	Tegaderm, Bioclusive, Blisterfilm, Omniderm, Proclude, Mefilm, Carrafilm, and Transeal
Foams	Bilaminate dressings with hydrophobic surface; suitable to mild/ moderate exudative wounds	Easily shaped to accommodate site of wound; prevent leakage of drainage and bacterial contamination	Can become adherent if drainage dries	Polymem, Allevyn, Biopatch, Curafoam, Flexzan, Hydrasorb, Lyofoam, and Mepilex

1.3.1 Topical antimicrobial dressing used in wound care

Chronic wound infections represent an increasing burden on healthcare systems worldwide. However, there is a lack of evidence regarding the use of antibiotics and antimicrobial dressings in the management of this burden, including its effectiveness and the best regimens for treatment (NICE, 2016). Based on expert opinion, many recommendations are present regarding the avoidance or use of antibiotics for chronic wounds (Gottrup et al., 2014). Douglas and Simpson (1995) support the early use of antibiotics in the presence of clinical signs of infection (Douglas and Simpson, 1995). Moreover, Robson and Barbul, have recommended that the use of systemic antibiotics must be restricted only to cases with systemic infection, acute foot infections and local cellulitis (Robson and Barbul, 2006). Systemic antibiotics do not effectively reduce the number of bacteria in granulating wounds compared to topically applied antibiotics (Diehr et al., 2007, Lipsky and Hoey, 2009, Siddiqui and Bernstein, 2010). The ability of topical antimicrobials to deliver high concentrations of antibiotics to wound sites could be the main reason for their effectiveness in chronic wound management (Lipsky, 1999). Further advantages include the lack of adverse systemic effects and have traditionally been associated with limited reports of resistance (Williamson et al., 2017), although it must be noted that more recent observations have sounded concern regarding the use of topical antimicrobials and the development of antimicrobials resistance (Bessa et al., 2016, Harkins et al., 2018, Percival et al., 2005).

Frequent use of topical antimicrobial agents reduces the availability of sensitive strains and supports resistant strains to increase prevalence. For example, mupirocin-resistant strains were observable in 100 different countries where mupirocin, a topical antibiotic useful against wound infections such as impetigo, was available. However, mupirocin- resistant organisms have not been detected in Norway, where mupirocin was not authorized (Gottrup et al., 2014). The continued emergence of antibiotic-resistant strains has enhanced the urgent need for an

alternative treatment to chronic wounds (Davies, 2003). Topical antimicrobial agents other than antibiotics have recently been used as an alternative for topical wound treatment and have broader spectrum antimicrobial activity than antibiotics (Bigliardi et al., 2017).

There are multiple varieties of antimicrobial dressings that are used in wound care, including silver, iodine, and honey. However, there is limited high-quality data to support their use. Recent clinical guidance and Cochrane reviews suggest that the regular use of antimicrobial dressings for the management of chronic wounds is not supported by scientific evidence (Vermeulen et al., 2007, Jull et al., 2015a, Health and Excellence, 2014, NICE, 2016). Most trials on dressings were considered to be poorly conducted due to several limitations, such as poor study design and high risk of bias, small sample size, lack of blinding, and the absence of significant clinical outcomes (Bradshaw, 2011, Halstead et al., 2015, Valle et al., 2014). Despite this lack of evidence, the NHS spent around £110 million yearly on advanced antimicrobial dressings (NICE, 2016).

Choosing an appropriate dressing can help in wound healing and should depend on a careful examination of the wound and the patient's general health and preferences (Metcalf et al., 2017). Maillard and Denyer (2006) have suggested a list of characteristics that the ideal antimicrobial dressing should have including continuous antimicrobial activity, comfortable, maintains the moisture of wound bed, affords an effective microbial barrier, treats exudate, and prevents wound trauma on removal (Maillard and Hartemann, 2013).

1.3.1.1 Iodine

Iodine is a natural non-metallic, dark violet element that plays a key role in human metabolism. Iodine is found naturally as iodide ions within the seawater, certain water weeds, fish, and oysters. It has been described as 'the most potent antiseptic available (Burks, 1998). Iodine's exact antimicrobial mechanism of action is not fully understood but is believed to be

associated with its ability to rapidly penetrate through the cell wall of microorganisms where it may oxidize nucleotide fatty/amino acids present in the bacterial cell membrane (Kanagalingam et al., 2015, Burks, 1998). It is indicated to prevent wound infection or recurrence of infection in patients at significantly increased risk of infection (Burks, 1998).

The task of evaluating the choice of povidone-iodine solution for the treatment of wounds is made complex by two factors. First, despite the existence of a large body of research inquiring into the use of povidone-iodine in wound care, the combination of laboratory, animals and human experiments, often using different preparations, is confusing (Selvaggi et al., 2003). Second, the relevance of in vitro studies regarding safety and effectiveness to in vivo use with patients may be limited. Mostly, published research on the issue of wound healing utilises animal models; but the applicability of the animal wound healing to the human's wounds remains questionable, as human patients often have underlying medical conditions that complicate healing (Lipsky and Hoey, 2009, Bigliardi et al., 2017).

The advantages of iodine as an antimicrobial agent include high potency, minimal adverse effects, and broad-spectrum range of action and ease of application. However, it suffers from some serious limitations which comprise cytotoxicity after prolonged application and delaying healing of the wound (Burks, 1998, Bigliardi et al., 2017).

1.3.1.2 Silver

Silver and silver nanoparticles (AgNP) exhibit inhibitory and bactericidal effects and thus, have been utilised for the treatment of skin ulcers, bone fractures and supporting chronic wound healing throughout history (Wilkinson et al., 2011). The application of silver in clinical practice takes the form of silver sulfadiazine or silver nitrate. Silver nanoparticles entered clinical practice as both an antibacterial and antifungal for more than 100 years and have been registered in the United States as a biocide material since 1954 (Nowack et al., 2011). They are

defined by their size (10-100nm) and their unique physiochemical properties (Wilkinson et al., 2011). Given their nanostructure, they exhibit an increase in its surface area to volume ratio when compared to ionic silver, therefore more numbers of atoms are available on the surface. Nanosilver is now applied in the coating of medical devices, including catheters, and as a component of wound dressings. The unique properties of nanoparticles have been shown to be advantageous in various medical fields, however, potential interactions with biological systems and toxicity have also raised concerns with regards their use in humans (Wilkinson et al., 2011).

Few randomized controlled trials support the use of silver dressings, however, the meta-analyses in (VULCAN trial) established that silver dressings were combined with the additional cost of 97 pounds in comparison with another type of dressing (Michaels et al., 2009). Interestingly, silver dressings were associated with approximately 18% (20.5 million pounds) of the total charge of advanced wound dressings (NICE, 2016, Hussey et al., 2019). Consequently, the BNF declares that silver dressings should only be used when clinical symptoms or signs of infection are apparent (NICE, 2016).

The key advantage of silver dressings in the prevention of recurrent wounds due to the antibacterial barrier it creates once released on the wound (Silver et al., 2006). Silver ions have been shown to penetrate the cell and cause damage to cellular contents such as DNA damage, disruption of the cell membrane and stimulation of antioxidant enzymes (McShan et al., 2014). However, the key disadvantage is the risk of toxicity due to heavy metal poisoning (Silver et al., 2006). Arora et al. and Hussain et al found that nanoparticles cause toxicity in mouse liver cells. They noticed the irregular cell shape and changes in the size of mitochondria of the exposed liver tissues (Arora et al., 2009, Hussain et al., 2005). Additionally, resistance to silver has been reported, mainly with gram negative bacteria (Finley et al., 2015a, Finley et al., 2015b).

1.3.1.3 Honey-impregnated dressings

The physical characteristics of honey alone can positively influence the wound healing atmosphere and the process of healing due to its acidity and the pH of about 3.2-4.5 (Molan and Rhodes, 2015). It is also well known that topical acidification of wounds promotes healing by increasing the release of oxygen from haemoglobin (Kaufman et al., 1985). In addition, this pH is less favourable for protease activity, thus, reducing the destruction of the matrix needed for tissue repair (Greener et al., 2005). The high osmolarity of honey due to its high sugar level is also useful to the process of healing, as substantiated in studies indicating sugar pastes to be effective as wound dressings (Biswas et al., 2010). Intrinsically, the osmotic effect of the sugar draws water from the wound bed and, though it could be thought that this may potentially harm and dehydrate the wound tissue, this is not the case. If the circulation of underneath blood to the wound is enough to replace the lost fluid from cells, then the surface's osmotic effect of sugar would simply create an outflow of lymph (Chirife et al., 1982). Such an outflow is favourable to the healing process, as it can be validated by the negative pressure wound therapy (Molan and Rhodes, 2015). Additionally, sugar draws water out of microbial cells, hence inhibit the growth of bacteria as long as the sugar does not become too diluted by the wound exudate (Topham, 2000).

The antibacterial activity in most of the honey beyond that which is due to acidity and osmolarity is due to hydrogen peroxide (Molan, 1992a). It is produced by an enzyme called glucose oxidase that bees add to the collected nectar in honeycombs. This enzyme becomes active only if the honey becomes diluted, as with wound exudate (Bang et al., 2003). However, it will be less potent in a wound environment since wound tissue comprises the enzyme catalase that breaks down hydrogen peroxide (Schepartz and Subers, 1964, Weston, 2000, Bang et al., 2003). Honey from Manuka trees has an exceptional type of antibacterial activity which is purportedly based on methylglyoxal, and as such it is not affected by the catalase enzyme in

wounds (Molan and Rhodes, 2015). Methylglyoxal, is a small and reactive molecule that forms by spontaneous conversion from its precursor substance dihydroxyacetone that is found in Manuka honey (Adams et al., 2009). Methylglyoxal is a cytotoxic compound, and the possibility of its contribution to delay wound healing in diabetic patients has been raised (Majtan, 2011). However, it would appear that the combination with other elements in the Manuka honey counteracts such toxicity (Kalapos, 2008, Blair et al., 2009). In addition, clinical trials using Manuka honey dressing on non-healing diabetic foot ulcers indicate that rapid healing within 3 months is achieved (Molan and Rhodes, 2015).

An optimal honey dressing would be one that can provide constant exposure of the wound with honey and absorb wound exudate. This aim can be achieved by the usage of primary dressings in which the honey is impregnated in a permeable material. There are three various types of such dressings; 1) honey incorporated to alginate fibre dressing which transforms to gel as soon as contact with wound fluid. The main disadvantage of this type of dressing is the limitation in the capacity to absorb fluid. 2) honey impregnated to polyacrylate gel that can absorb an adequate amount of wound exudate and swell. 3) honey impregnated into a primary dressing composed of superabsorbent fibres that have a high capacity to absorb the mixture of honey and wound fluid from the interstitial spaces and provide continuous diffusion of honey into the wound area (Molan and Rhodes, 2015).

Despite the various properties of honey with a positive effect on wound healing, there are some possible adverse effects on the use of honey in wound care. In some cases, a burning sensation can arise due to the acidity of honey (Sharp, 2009). Allergic reaction to honey is another possible side effect, however, this is rare because honey prepared for use in wound care is passed through a filter with the purpose of removing pollen (Molan, 2001). Additionally, dehydration of the wound can occur with excessive use of honey due to the osmotic effect but adding a physiological solution such as oil on the dressing prevents

dehydration (De Rooster et al., 2008). Resistance to honey seems to be less likely due to its multiple antimicrobial target sites. Several studies have found that bacteria unlikely developing resistance to honey, even with long exposure to sub-inhibitory concentrations (Blair et al., 2009, Cooper et al., 2010a). However, in 2014, Camplin and Maddocks demonestrated that *P. aeruginosa* treated with Manuka honey within a recognized biofilm can acquire resistance and increase capability to biofilm formation (Camplin and Maddocks, 2014).

1.3.2 Evidence for the use of honey in the management of chronic wounds

Several animal studies and randomized control clinical trials have provided strong evidence that honey can augment wound healing (FUJII et al., 1990, Siavash et al., 2015, Al-Waili et al., 2011, Efem, 1988). The reported trials have variable quality and currently the evidence suggests to only encourage the use of honey in the treatment of mild-to-moderate superficial and partial thickness burns (Jull et al., 2015b). A study conducted by Jull and co-workers demonstrated that honey-impregnated dressings, compared to standard of care, did not significantly help in venous ulcer healing at 12 weeks. Furthermore, these dressings did not show any improvement in ulcer size, time to healing, the occurrence of infection and quality of life (Jull et al., 2008). On the other hand, two other studies suggest that a clinically significant reduction in healing time and incidence of infection was observed after treatment with honey compared to conventional treatment. However, the number of patients recruited for these trials was insufficient to show statistical significance (Gethin and Cowman, 2009, Robson et al., 2009). The National Institute for health and care excellence (NICE) reviewed the evidence for different types of antimicrobial dressings, including honey and concludes that there is limited high quality data to support the use of such interventions (NICE, 2016).

1.4 Biofilms

A biofilm is a complex environment of microorganisms in which microbial cells attach to each other on a living or non-living surface within a self-formed extracellular polymeric matrix (McKay and Nguyen, 2017). This matrix is a key element in biofilm formation and is composed mainly of water, cells and secreted macromolecules from inhabitant microorganisms (Gilbert et al., 2002b). The biofilm matrix is formed to protect bacteria from various environmental stresses, such as nutrient depletion, desiccation, altered pH or exposure to sub-inhibitory concentrations of antimicrobial (Gambino and Cappitelli, 2016). Biofilms are commonly found as polymicrobial whereby they contain multiple diverse species, but can also be found as single fungal or bacterial species (Koo et al., 2017). They are a major contributor to diseases that are characterized by chronic inflammation and an underlying bacterial infection, such as chronic wounds (James et al., 2008, Omar et al., 2017, Malone et al., 2017). Therefore, chronic wounds are often resistant to common treatments such as conventional antibiotics or some antimicrobial wound dressings like silver (Mah and O'Toole, 2001).

1.4.1 Biofilm formation

Biofilm formation is a multistep system, and it differs between bacterial species. There are mainly five consecutive stages to illustrate the formation of biofilm including, initial reversible attachment, irreversible attachment, microcolony formation, biofilm maturation, and detachment (Sauer et al., 2002, Stoodley et al., 2002). A schematic diagram of microbial biofilm development is shown in Figure 1.1.

Stage 1: Motile cells reversibly attach to the surface either by physical forces such as the van der Waals or stereotactic forces or by microbial appendages like flagella or pili (Marić and

Vraneš, 2007). The bacterial adhesion can be greatly modulated by different factors, such as surface functionality, temperature, and pressure (Garrett et al., 2008).

Stage 2: Some of the reversibly attached cells remain immobilized and adhere irreversibly if the attractive forces exceed repulsive forces (Garrett et al., 2008). The physical appendages of bacteria such as flagella and pili have been documented to overcome the initial electrostatic repulsive forces and consolidate the interactions between bacteria and the surface (Kumar and Anand, 1998). The hydrophobicity of the cell surface also play a key role in biofilm formation when the bacteria adhere to a hydrophobic nonpolar surface since this interaction reduces the repulsive force between them (Tribedi and Sil, 2014).

Stage 3: During this phase, microbial cells begin to communicate among themselves by quorum sensing (QS) via the production of autoinducer signals which result in the expression of biofilm-specific genes (Davies et al., 1998, Vasudevan, 2014). Also, bacteria start to produce the extracellular polysaccharide matrix to protect the biofilm network from mechanical damage and shear that results from fluid flow. The extracellular polymeric substances (EPS) are composed of polysaccharides, proteins, lipids, and DNA (Tuson and Weibel, 2013). It has been stated in this context that *P. aeruginosa* produces and releases three polysaccharides, named alginate, pel and psl, which give the biofilm stability. Alginate interacts with water and nutrients, which provides the biofilm with nutrients, while pel (glucose rich polysaccharide) and psl (pentasaccharide) act as a scaffold to biofilm structure (Rismondo et al., 2016, Franklin et al., 2011). Additionally, extracellular DNA (eDNA) was also reported to be responsible for cellular communication and stabilization of *P. aeruginosa* biofilm (Gloag et al., 2013). At this stage, the biofilm is multi-layer and its thickness is increased to 10 µm (Gupta et al., 2016).

Stage 4: the microcolony grows in size and reaches a thickness of around 100 µm. Microcolonies in biofilm frequently consist of diverse microbial communities. The micro-

consortia of these multispecies work in a fairly complex and organised manner. Its close proximity increases the exchange of substrates, the delivery of metabolic products, and the elimination of toxic end products (Davies and Marques, 2009, Gupta et al., 2016). In this stage of maturation, biofilm is adopted with the external environment by controlling its structure, physiology, and metabolism. Thus, the third and fourth stages involve the aggregation of cells, the formation of microcolonies followed by the growth and maturation of the adhered cells (Gupta et al., 2016).

Stage 5: this stage is the dispersion that marks the release of the biofilm and the return of the sessile cells to the motile form (Hall-Stoodley et al., 2004). Once the availability of nutrients is reduced, small clusters of cells detach themselves from the stacks and migrate towards a new nutrient-rich environment, enabling the cells to travel in a new biofilm cycle (Costerton et al., 1995, Gupta et al., 2016). In this process, different saccharolytic enzymes are produced by the microbial community inside the biofilm that breaks the biofilm stabilizing polysaccharide and thus release surface bacteria residing on top of the biofilm structure for colonization into a new region. For example, *Pseudomonas* spp release alginate lyase, *Escherichia coli* releases N-acetyl-heparosan lyase, and *Streptococcus* spp produce hyaluronidase enzymes for the breakdown of the biofilm matrix (Sutherland, 1999). Additionally, the expression of flagella proteins is upregulated by microorganisms so that the organisms become mobile and the bacteria can move to a new site (Otto, 2013).
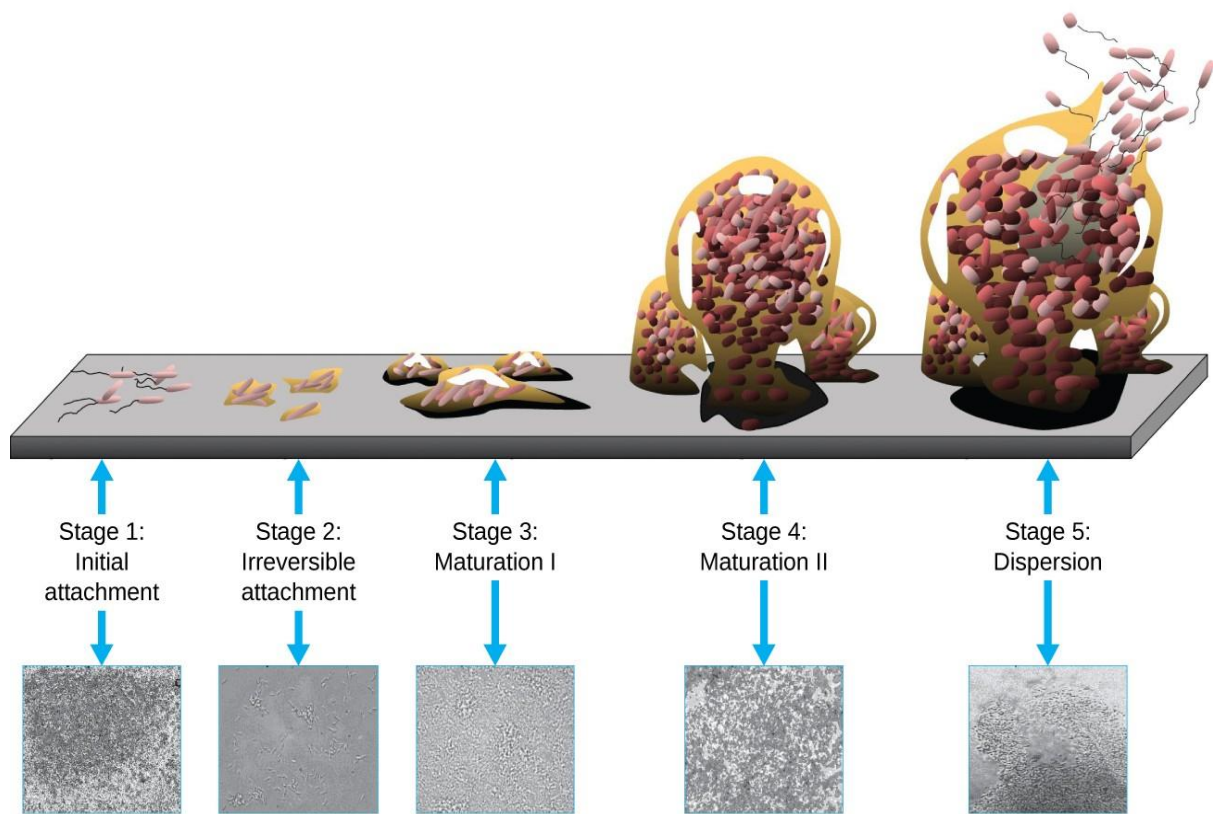
Figure 1. 1 The five stages of biofilm formation, taken from (Monroe, 2007). Biofilm life cycles sequentially involve reversible adhesion, irreversible adhesion, maturation I (microcolony formation), maturation II, and dispersion. Each cartoon is paired with photomicrographs, taken on the same scale of the stage. Notice that the bacteria's micrographs for reversible adhesion and dispersion are comparable because bacteria are essentially in a planktonic state, whereas the micrographs representative of irreversible adhesion and maturation II indicate that the bacteria are becoming progressively organized.

1.4.2 Biofilm insusceptibility to antimicrobials

Biofilm infections can be difficult to treat with antimicrobials as a 100 to 1000-fold increase in antimicrobial tolerance has been reported in biofilms when compared to planktonic cells (Ceri et al., 1999). There is no single mechanism for biofilm recalcitrance to antimicrobials and numerous mechanisms likely function in consortia to achieve this characteristic (Figure 1.2).

1.4.2.1 Reaction-diffusion limitation

Antimicrobials may be prevented from entering the biofilm and reaching their target sites by its matrix acting as a barrier. The extracellular polymeric substances (EPS) can represent 90% of the biofilm structure and generally composed of polysaccharide, DNA, and proteins. However, its exact composition is often poorly described and can vary greatly dependent on the strains, species, and growth conditions (Branda et al., 2005). Although penetration prevention is no longer considered a significant factor, antibiotics may not penetrate if they bind to components of the biofilm matrix or to bacterial membranes (Walters et al., 2003, Chiang et al., 2013). For instance, aminoglycosides and polypeptides are positively charged antibiotics that bind to negatively charged biofilm matrix polymers are delayed in their penetration through biofilm (Olsen, 2015). In terms of *P. aeruginosa* biofilm, the EPS alginate produced by *P. aeruginosa* was studied in depth because of its ability to trap antimicrobials, an ability that is believed to be due to its strong anionic nature (Alkawash et al., 2006, Gordon et al., 1988). Alginate-overproducing strains have developed biofilms more resistant to aminoglycoside treatment than wild-type strains in several laboratory biofilm models, indicating that alginate can inhibit aminoglycoside diffusion (Gordon et al., 1988, Alkawash et al., 2006). Furthermore, exogenous DNA may induce resistance to cationic antimicrobial peptides and aminoglycoside in *P. aeruginosa* by sequestration of cation and by modification of PhoPQ/ PmrAB-mediated LPS (Mulcahy et al., 2008). This eDNA principally provides a structural role and as such infers biofilm stability, but may also act in the binding of cationic antimicrobials through charge effects and as such may also play a role in drug recalcitrance (McKay and Nguyen, 2017).

1.4.2.2 Physiological gradients

The ability of antibiotics, such as beta-lactams, to kill bacteria has long been related to the bacterial growth rate (McKay and Nguyen, 2017). In the biofilm, multiple microcolonies form a metabolically heterogeneous bacterial population (Hall-Stoodley et al., 2004). Bacteria deep inside the biofilm experience a lack of nutrients and oxygen, and a build-up of waste products such as carbon dioxide. Hence, they show a reduction in their metabolic activity that in some cases may be characterised by a cessation of growth (Dunne, 2002, Donlan and Costerton, 2002). Nutrient-deficient zones may produce stationary phase-like dormant cells with reduced sensitivity to antibiotics (Walters et al., 2003). In addition, cells with diverse phenotypes and genotypes coexist within the biofilm population. This indicates that distinct metabolic pathways are expressed based on local environmental conditions in the biofilm (Walters et al., 2003, Pamp et al., 2008). Many antibiotics target processes that occur in growing bacteria, such as replication, transcription, translation, and synthesis of cell walls. Therefore, enhanced antimicrobial recalcitrance may occur in low metabolic activity biofilm bacteria found in the inner section of the biofilm (Ciofu et al., 2015).

1.4.2.3 Presence of persisters

Persisters are a small bacterial subpopulation, estimated to constitute 0.1%-10% of biofilm, that have reached a slow-growing or starving state and are by definition able to survive high concentrations of antimicrobial (Lewis, 2012, Hu and Coates, 2012). The reduction in their metabolic rates make them less susceptible to antimicrobials, however, studies have shown that persisters can withstand treatments with bacteriocidal concentrations of antimicrobials that can destroy non-growing bacteria (Lewis, 2012, Kaldalu et al., 2004). Therefore, the reduced growth rate alone cannot account for this increased resistance (Lewis, 2005). Various processes

have been involved in persister development and have recently been thoroughly studied (Wood et al., 2013, Gerdes and Maisonneuve, 2012, Balaban, 2011, Lewis, 2010). The primary model for persisters development includes the expression of toxin-antitoxin (TA) modules. These usually include a stable toxin protein which interferes with an important cellular process and a labile antitoxin (protein or RNA) which prevents toxicity. Antitoxin degradation or overexpression of toxins beyond their corresponding antitoxins causes a state of dormancy (Wood et al., 2013, Gerdes and Maisonneuve, 2012, Balaban, 2011).

1.4.2.4 Stress responses

Several stress responses are activated during the growth of biofilms, which can in turn regulate cellular pathways that confer antibiotic resistance and tolerance. These stress responses may be caused by environmental signals that prompt the transition from planktonic to biofilm lifestyles or by the microenvironment generated by biofilm growth (McKay and Nguyen, 2017). Universal stress genes are more strongly expressed at the top of a thick biofilm, while slow-growing cells deep in the biofilm experience little expression of hypoxia-regulated genes, most likely due to long-term anoxia (Williamson et al., 2012). In mature *E.coli* biofilms, increased resistance to antibiotics and the development of ampicillin-resistant subpopulations in the deeper layers have been promoted by the rpoS-mediated stress reactions (Ito et al., 2009). The SOS stress response in heterogeneous microenvironments and nutrient-deprived biofilm induced biofilm-specific high resistance to the fluoroquinolone ofloxacin (Bernier et al., 2013). It is also thought that oxidative compounds in the biofilm promote the overexpression of certain efflux proteins that are involved in the extrusion of antibiotics from bacteria and thus trigger antimicrobial resistance (Jolivet-Gougeon and Bonnaure-Mallet, 2014).
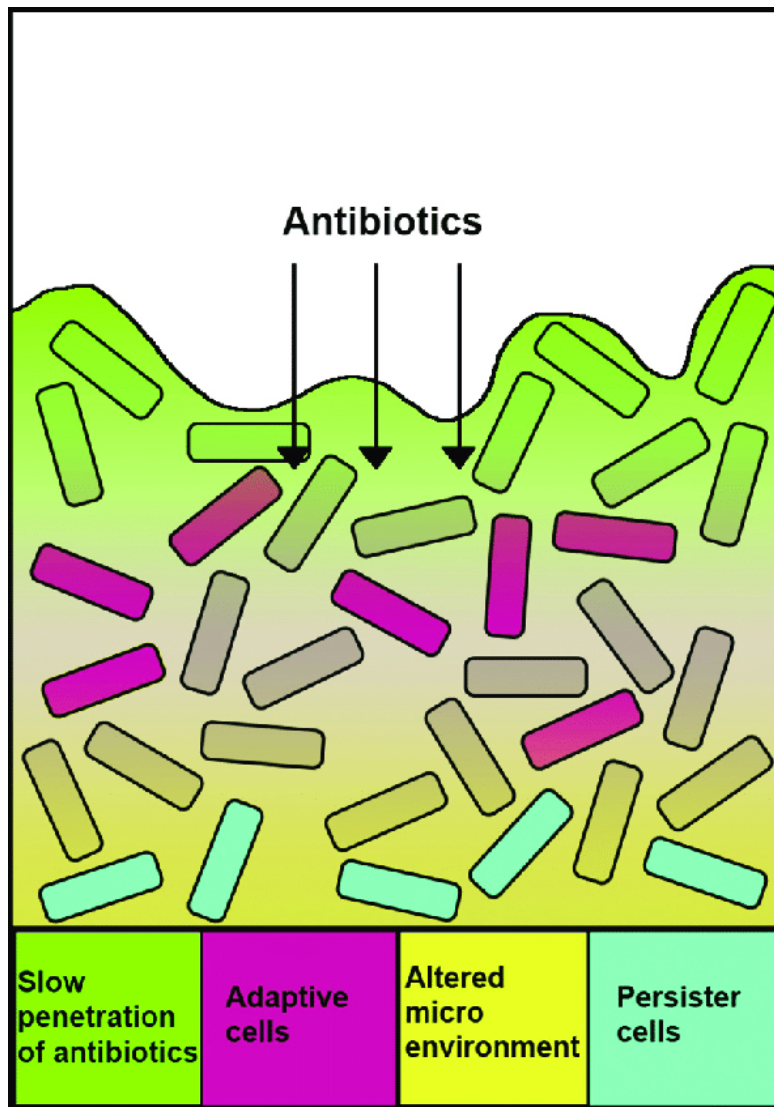
Figure 1. 2 Mechanisms of biofilm-mediated antibiotic resistance, Taken from (Pang et al., 2019). Antibiotics slowly enter the biofilm (green); some biofilm cells express adaptive response to survival under harsh conditions (Purple); the altered chemical microenvironment (yellow) inside the biofilm induces slow growth of bacteria which reduces antibiotic uptake; multidrug-resistant persister cells are formed (blue).

1.4.3 The impact of bacterial biofilms on chronic wounds

In chronic wounds, bacterial biofilms have now become identified. Early proof of bacterial biofilms in wounds was obtained from experimentally induced chronic animal wounds and subsequently seen in clinical wounds. Biofilms can be detected microscopically in up to 60% of chronic wounds but in just 6% of acute wounds (James et al., 2008). *Staphylococcus* spp and

Pseudomonas spp were the most frequently isolated biofilm-forming bacteria from chronic wounds (James et al., 2008). Evidence suggests that biofilm plays a major role in the failure of chronic wounds to heal (Rhoads et al., 2007, Zhao et al., 2010, Phillips et al., 2008, Bjarnsholt, 2013). Pro-inflammatory cytokines released by necrotic tissue, foreign material, and microorganisms enable the continuation of the inflammatory stage (Cornell et al., 2010). Additionally, fibroblasts and keratinocytes, the essential cells in the wound healing steps, are phenotypically altered in the setting of chronic wounds so that their ability to replicate and to generate the required building blocks for granulation tissue formation is altered (Clark, 2008, Morasso and Tomic-Canic, 2005).

Chronic biofilm diseases are recurrent and difficult to remove. They respond moderately to antibiotics prescribed based on the results of minimum inhibitory concentration (MIC) tests and may recur once the antibiotic course is complete (Kadurugamuwa et al., 2003, Sethi and File, 2004, Wolcott and Ehrlich, 2008, Sanchez et al., 2013). In a clinical study, three patients with large non-healing venous leg ulcers that contain high proportions of *P. aeruginosa* in their wound biofilm were debrided using standard sharp debridement methods. Surgical debridement of those chronic wounds effectively eliminated biofilm communities from wound beds, however, biofilms started to re-emerge within 2 days from the initial debridement (Wolcott et al., 2010). This suggests that, after debridement, there is a period of opportunity during which the planktonic bacteria recolonizing the wound bed are vulnerable to treatments that can effectively destroy them and prevent biofilm communities from reforming (Omar et al., 2017).

1.5 Medical-grade Manuka honey

Medicinal honey research is experiencing a substantial renaissance. It has passed through a transition from a traditional remedy which has been largely dismissed by conventional medicine as "alternative" (Jenkins and Cooper, 2012). Recently, the rise in antibiotic resistance by many bacterial pathogens has impelled interests in evolving and using novel non-antibiotic antimicrobials (Allen et al., 2014a). Honey is often only used as a last resort for treating chronic wounds, usually, after conventional antibiotic treatments have failed (Simon et al., 2009, Mandal and Mandal, 2011). Honey is widely considered non-toxic to humans (Mohamed et al., 2015, Abdelatif et al., 2008) and has been applied in wound dressings or gels to treat ulcers and burns (Alcaraz and Kelly, 2002, Molan, 2002). A variety of clinical case studies have demonstrated superior *in vivo* activity of honey to facilitate wound healing (Molan, 2002, Molan, 2006, Molan and Rhodes, 2015). Despite all the advantages of using honey in chronic wounds, double-blinded clinical trials are lacking in the literature (Molan and Cooper, 2000, Cooper et al., 2001).

The vast majority of current research has concentrated on honey's antimicrobial properties, although there is a growing body of research indicating that many varieties have other mechanisms of action that modulate the wound healing process (Molan, 2006, Lee et al., 2011b, Lee et al., 2011a). Variability in the concentrations and composition of the bioactive components of different honey varieties indicates that different types of honey have different medicinal properties, or in other words, not all honeys function similarly (Allen et al., 1991, Cooper and Jenkins, 2009, Kwakman et al., 2011b) Although over 300 forms of honey are found worldwide, most studies have been conducted on Manuka honey (Tsang et al., 2018). Manuka honey is a native product of New Zealand and Australia; produced by bees that pollinate the Manuka bush (Figure 1.3) (Carter et al., 2016b). Peter Molan, a professor at Waikato University, New Zealand, is acknowledged to be the first scientist to report on the

unique activity of Manuka honey; and began determining its potency against a broad range of diverse bacterial species in the mid-1980s. However, while it was clear that even low amounts of Manuka honey cleared bacterial pathogens, the precise active ingredient accountable for this action remained elusive (Molan and Russell, 1988, Molan and Cooper, 2000, Molan, 1992a, Molan, 1992b, Molan, 2002, Willix et al., 1992).
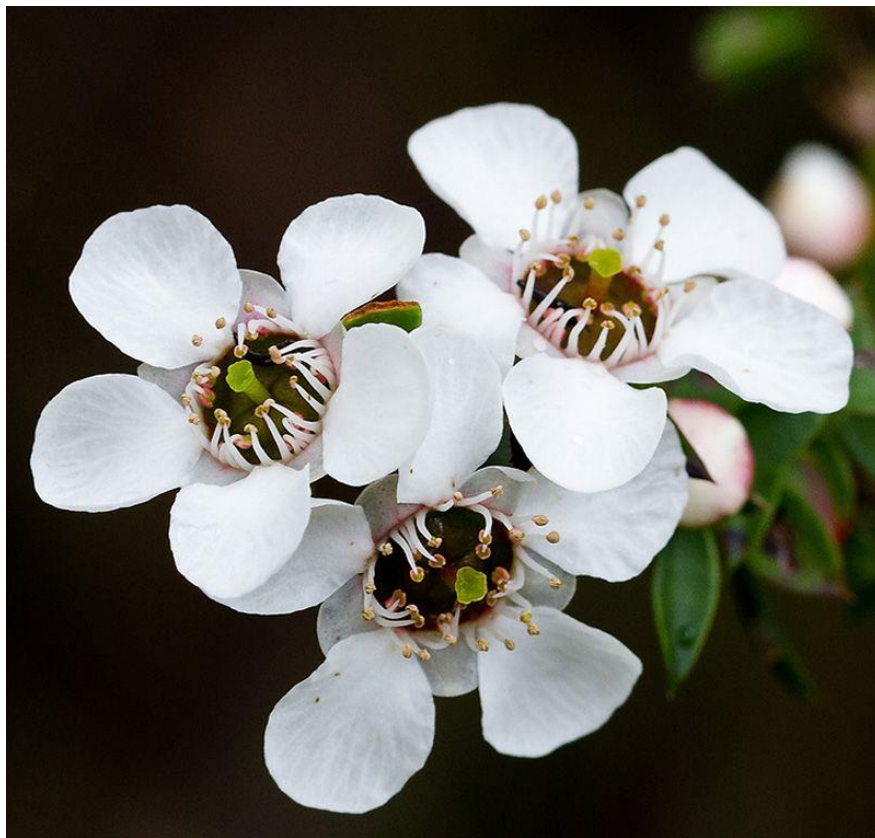
Figure 1. 3 Manuka flowers. Image taken from (https://www.shutterstock.com). The popular Manuka honey is made from *Leptospermum scoparium*, which is native to New Zealand and parts of Australia. It has strong antibacterial and anti-inflammatory properties.

1.5.1 Antibacterial mechanisms of Manuka honey

The antibacterial properties of honey varies depending on floral source, geographic location, or storage conditions (Molan and Cooper, 2000). Moreover, it is reported that the bactericidal effect of honey depends on the concentration of honey used and the nature of the bacteria (Basualdo et al., 2007, Adeleke et al., 2006). Honey has three essential properties that contribute to its antibacterial function. Namely low water activity, low pH and the activity of hydrogen peroxide after dilution (Mavric et al., 2008, Kwakman et al., 2011a, Stephens et al., 2010).

Honey is a complex compound with more than 150 components, mainly consisting of different types of sugar, minerals, acid, proteins and only about 17% water. The high osmolarity results in low water activity (a_w), with an average of 0.6 of undiluted honey (Chirife et al., 1983). At a water activity of 0.94-0.99, many bacterial species have optimum growth and therefore cannot grow in undiluted honey (Lambert, 2003, Blickstad, 1984). Generally, planktonic microorganisms are capable of survival in concentrations of honey below 2-12 % or through sporulation (Scott, 1957, White, 1996, Olaitan et al., 2007). When honey is diluted, water activity would increase and therefore the osmolarity is no longer an inhibitory factor (Kwakman and Zaat, 2012). In addition, the sugar component of honey would be readily diluted by wound exudate in the wound environment (Molan and Betts, 2004). Thus, high osmolarity and low water activity cannot be considered as a stable antibacterial factor (Kwakman and Zaat, 2012).

The acidity of honey is the second common antimicrobial property. The pH of honey is usually between 3.2-4.5 (Simon et al., 2009, Mandal and Mandal, 2011). This acidity is mainly due to the nectar ripening and when gluconic acid is formed from a glucose enzyme reaction with glucose oxidase. The growth of certain common microorganisms is assumed to be

inhibited by acidity (Molan, 1992b). For instance, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae* cannot grow when the pH is below 4 (Molan, 1992b, Simon et al., 2009). It is unlikely, however, that pH is the key component of honey antibacterial activity, as the low pH was not revealed after the neutralization of other significant antibacterial components of honey (Kwakman and Zaat, 2012).

Hydrogen peroxide is the most widely recognized antibacterial component of a variety of honeys. This antibacterial agent is produced enzymatically from glucose when diluted in the presence of glucose oxidase under aerobic condition, and it kills bacteria by oxidizing the cellular components (White, 1962, Finnegan et al., 2010).

Glucose oxidase

Glucose + water --------------------------» Gluconic acid + Hydrogen peroxide
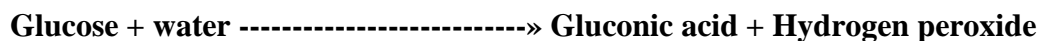
Glucose oxidase becomes inactive when free water becomes low in honey, but is active when the honey is diluted, as with wound exudate (Bang et al., 2003). Hydrogen peroxide production is at its maximum level when diluted between 30 and 50%, but its activity is significantly reduced at concentrations below 30% (Bang et al., 2003). Furthermore, several factors can reduce the hydrogen peroxide activity such as heat, UV light and filtration process occurring during the manufacture of commercially made honey products (Bang et al., 2003, Chen et al., 2012). Finally, the addition of the enzyme catalase, which can be produced by some bacteria such as Gram-negative ones as a protective mechanism against oxidative damage by reactive oxygen species, also neutralizes hydrogen peroxide (Zamocky et al., 2008). However, the amount of catalase provided by such bacteria is not considered to be sufficient to neutralize hydrogen peroxide in the honey (Molan, 2006).

1.5.2 The non-peroxide antibacterial activity of Manuka honey

Antibacterial activity that persists in honey after hydrogen peroxide neutralization is generally referred to as "non-peroxide" activity (NPA). NPA is equivalent to "Unique Manuka Factor" (UMF), a trademark registered with the UMF Honey Association and available for use by New Zealand Manuka honey producers under licence. NPA was first established in New Zealand Manuka honey and closely related to the floral source as it is usually obtained from phytochemical components produced by Manuka bush (Allen et al., 1991, Molan and Russell, 1988). The advantage of non-peroxide antibacterial activity is that it stays constant during long periods of storage of the honey and does not change with various heat and light conditions (Junie et al., 2016, Molan and Allen, 1996).

Methylglyoxal (MGO), which forms from the nectar-derived compound, dihydroxyacetone (DHA) during the ripening of honey, was known as the compound predominantly responsible for NPA of Manuka honey (Adams et al., 2008a, Adams et al., 2009). MGO (Figure 1.4) is the aldehyde form of pyruvic acid and itself is cytotoxic to both eukaryotes and prokaryotes (Együd and Szent-Györgyi, 1966a, Freedberg et al., 1971). Its primary objective is protein synthesis, although further studies have found that MGO also kills cells by irreversibly destroying cellular macromolecules including DNA and proteins (Együd and Szent-Györgyi, 1966b). However, bacteria also produce MGO as a glycolysis by-product, which can neutralize the action of MGOs (Baskaran et al., 1989). The glyoxylase system that consists of two enzymes, glyoxylase I and II, and which is widely distributed among bacteria, can detoxify MGO (Sukdeo and Honek, 2008).

While Manuka honey is well-known to have MGO, not all honeys have MGO. For the first time in 2008, Mavric and colleagues identified the MGO component in Manuka honeys using High-Performance Liquid Chromatography (HPLC) and showed that they contained between

300-700 mg/kg. By contrast, numerous other types of honey, for example, runny honey, contain an insignificant amount of MGO (1-2 mg/kg) (Mavric et al., 2008). MGO was assayed for the inhibition of the growth of *S. aureus*, *P. aeruginosa*, *E. coli* and *S. pyogenes*, and results indicated that MGO was the major component responsible for the non-peroxide activity of Manuka honey (Adams et al., 2008a, Kilty et al., 2011, Maddocks et al., 2012). A controversial study was reported by Molan, showing that the antibacterial activity of MGOs in honey is lower than that of MGOs in water only. This was determined by looking into Manuka honey's antibacterial activity against an equivalent concentration of MGO in solution, thus, these results suggest other components within the honey may change the activity of MGOs (Molan, 2008). The ability to chemically describe the MGO content in honey samples is important as accurate quantification of any antimicrobial components allow us to understand how honeys kill or inhibit the growth of microorganisms (Lu et al., 2013).
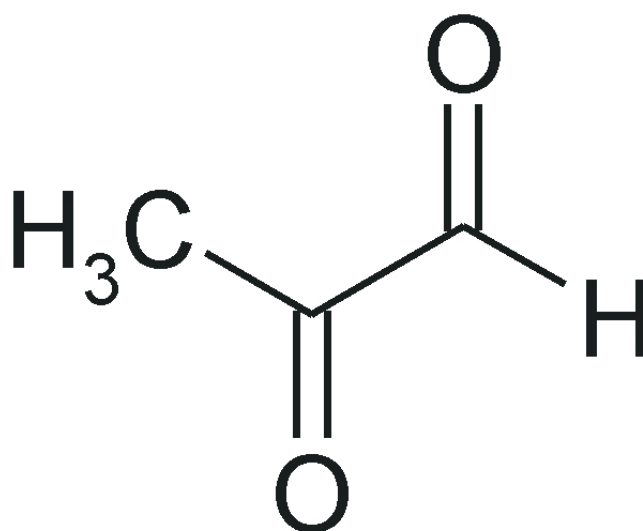
Figure 1. 4 Chemical structure of methylglyoxal (MGO), taken from (Laga, 2008).

Many antibacterial compounds in honey from different geographic regions have also been described. Bee defensin-1 is an antibacterial peptide that has been shown to display non-peroxide antimicrobial activity in the British honey Revamil (Kwakman et al., 2011a). Initially, bee defensin-1 was only found in Revamil honey and not any other honey samples tested. However, another study has shown that, in the one Manuka honey sample tested, bee defensin-1 does indeed exist (Weston et al., 2000). The bee defensin-1 appears to be modified in Manuka honey, and the presence of MGO masks its antibacterial activity (Majtan et al., 2012). The ability of MGO to react with lysine and arginine residues within proteins, including defensin, leading to their glycosylation and consequent inactivation (Poulsen et al., 2013).

Honey also consists of other components, such as phenolic compounds, flavonoids, and peptides, which together can contribute to their antibacterial activity (Stephens et al., 2010, Majtan et al., 2012). Studies have shown that phenolic compounds from plant nectar can significantly contribute to the non-peroxide antibacterial activity of honey (Molan, 1992b, Weston et al., 2000). Nevertheless, the function of these phenolic compounds in contributing to the overall antibacterial activity of the honey remains uncertain, as other antibacterial compounds, such as MGO and bee defensin, may also present and provide antibacterial properties (Maddocks and Jenkins, 2013). The determination of which of the compounds contribute to the bulk antimicrobial activity of each honey is complex and incompletely understood given the potential for interactions among the 200 known compounds that are reported to exist in honey (Eteraf-Oskouei and Najafi, 2013).

Finally, a recent analysis using Nuclear Magnetic Resonance (NMR) found an additional component in Manuka honey named Leptosin that may have antibacterial activity. Even though the biological activity and biosynthetic pathway/source of the glycoside (Leptosin) is still unknown, it may be a good chemical marker for Manuka honey's purity (Kato et al., 2012). The discovery of new honey components responsible for its antibacterial activity is

continuously progressing. Honey is generally a complex solution, containing multiple antimicrobial elements which target several active sites in microorganisms (Blair et al., 2009).

1.5.3 Effects of Manuka honey on bacterial cell structure and gene expression

The antimicrobial activity of honey is likely multimodal including direct destruction of microorganisms and anti-virulence function by inhibiting the gene associated with stress responses, secretion of virulence factors, and organisms multicellular functions like quorum sensing or formation of biofilm (Wang et al., 2012). Therefore, to determine the mechanism of actions of Manuka honey on bacterial cell growth and gene expression, proteomic and transcriptomic together with microscopic imaging have previously been conducted (Blair et al., 2009, Packer et al., 2012, Roberts et al., 2012a, Jenkins et al., 2011, Jenkins et al., 2014). Manuka honey has been approved to change the size and shape of bacterial cells and these changes vary in different bacterial isolates. For example, in *S. aureus* cultures, Manuka honey has been exhibited to interrupt the division stage of the cell cycle and lead to the formation of large cells containing septa as observed by electron microscopy, Figure 1.5 A (Henriques et al., 2010, Roberts et al., 2015). In addition, extensive structural damage was detected using an electron microscope in *P. aeruginosa* cells when exposed to minimum inhibitory concentration (MIC) of Manuka honey (Henriques et al., 2011). These changes in cell structures of *P. aeruginosa* were explained by quantitative PCR analysis that revealed a down-regulation of oprF (an outer membrane protein) which is essential in structural stability, Figure 1.5 B (Roberts et al., 2012a, Roberts et al., 2015).

Proteomics provides a group of techniques that can be used to analyse differences in protein expression between untreated bacterial cells and those that are treated with inhibitory concentrations of Manuka honey (Jenkins et al., 2014). In term of *S. aureus*, a study conducted

by Packer and his colleagues, using proteomics rather than transcriptomics, showed that *S. aureus* cells treated with a sub-MIC concentration of Manuka honey induced stress responses and altered protein synthesis (Packer et al., 2012). Another study examined the effect of Manuka honey on MRSA using molecular techniques such as two-dimensional electrophoresis and RT-PCR, and they found that multiple genes expressed differently after exposure to bactericidal concentration of Manuka honey. Genes with increased expression were involved in sugar, amino acid, and protein synthesis while those with decreased expression were associated with cell division, quorum sensing and virulence (Jenkins et al., 2014). However, any links observed in proteomic data when compared with gene expression data are not always acceptable and yet to be fully understood. In several investigations, the study of mRNA and protein expression data from the same cells under similar conditions failed to display a strong association between the two domains (Pascal et al., 2008, Gygi et al., 1999, Yeung, 2011, Ghazalpour et al., 2011). This discrepancy can be due to various factors such as different half-lives and post transcription machinery (Haider and Pal, 2013).

Regarding Gram-negative bacteria, *E. coli* respond to sub-MIC levels of Manuka honey via down-regulated gene expression in genes linked to protein synthesis and up-regulated gene expression in genes linked to stress responses (Blair et al., 2009). In *P. aeruginosa*, Manuka honey suppresses flagellar regulatory proteins (FleQ and FliA) causing a significant decrease in flagellated cells. As adhesion and cell motility are essential in *P. aeruginosa* virulence and biofilm formation, this observation may be of clinical significance (Roberts et al., 2014). As different bacterial species respond in a different way to Manuka honey exposure, more studies are needed to understand the mechanism of action of Manuka honey against a panel of bacteria.
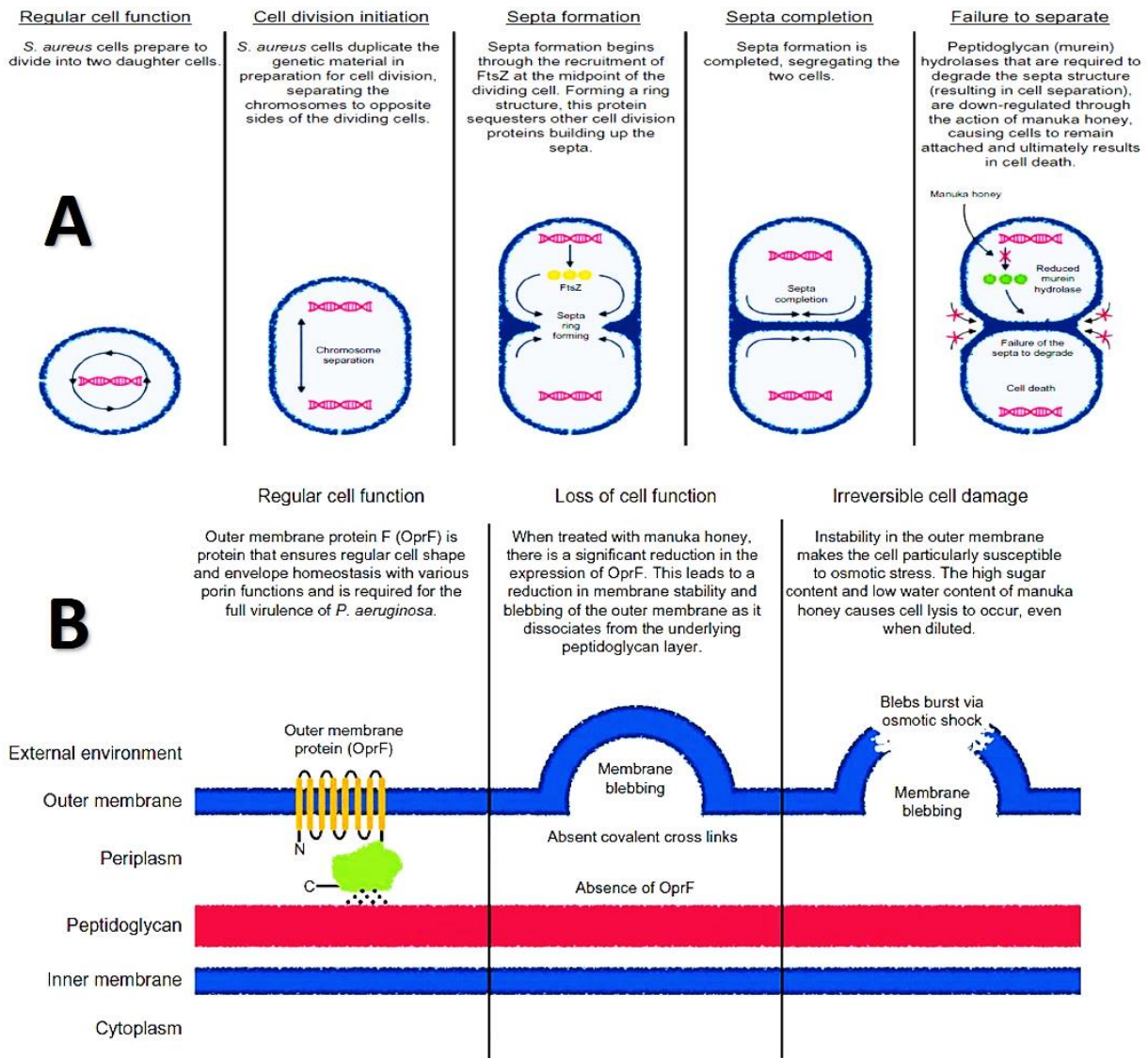
Figure 1. 5 The proposed mechanism of action of Manuka honey (Roberts et al., 2015). A) Manuka honey inhibits Methicillin-resistant *Staphylococcus aureus* (MRSA). Manuka honey is assumed to influence the final stages of cell division, after septa formation is completed, by reducing the production of peptidoglycan hydrolases. This leads to cell death because the septa cannot degrade, and the two daughter cells remain attach. B) Manuka honey inhibits *Pseudomonas aeruginosa* biofilms by down-regulating a key structural protein (oprF) that supports and maintains cell shape and cell envelop stability, this results in membrane blebbing and then cell lysis.

1.5.4 Antibiofilm properties of Manuka honey

Prolonged wound chronicity may be due to the formation of a bacterial biofilm within the wound where the bacteria remain covered by the biofilm matrix. The host immune system cannot clear these bacteria and show resistance to systemic as well as topical antimicrobial

agents (Edwards and Harding, 2004, Rahim et al., 2017, Vyas and Wong, 2016). Interestingly, several studies have demonstrated the effectiveness of Manuka honey to eradicate established and mature bacterial biofilms *in vitro*, although a higher concentration of honey is required to eradicate established biofilms than for planktonic cells (Okhiria et al., 2009, Maddocks et al., 2012, Majtan et al., 2014). Hammond et al. (2014) demonstrated a relationship between the concentration of Manuka honey applied and the reduction of biofilm biomass. Three *Clostridium difficile* strains were used in this study and each test strain was grown in sterile microtiter plates for 24 and 48h to allow biofilm formation. The effect of Manuka honey on mature biofilms was examined at different concentrations between 1% and 50% (w/v) of Manuka honey. Concentrations of medical-grade honey ranging from 20 to 50% (w/v) resulted in significant reductions in biofilm biomass produced by *Clostridium difficile* strains (Hammond et al., 2014). In a separate study, *in vitro* mature *Pseudomonas* biofilms treated by 40% and 20% (w/v) Manuka honey revealed that the mean absorbance of biofilm biomass was significantly reduced with exposure to 40% w/v Manuka honey compared to 20% w/v Manuka honey ($P<0.05$) (Okhiria et al., 2009). These studies support that the antibacterial and antibiofilm activities of Manuka honey depend on the concentration used and strains tested. Methylglyoxal seems to be primarily, but not completely responsible for inhibiting biofilms by Manuka honey, emphasising once again the importance of additional elements that regulate activity (Kilty et al., 2011, Lu et al., 2014).

Manuka honey prevents the formation of bacterial biofilms by disrupting cellular adhesion and aggregates in multiple Gram positive and negative pathogenic bacteria including *Staphylococcus* and *Streptococcus* species, *Escherichia coli*, *P. aeruginosa*, *Enterobacter cloacae*, *Proteus mirabilis* and *Klebsiella pneumoniae* (Maddocks et al., 2012, Maddocks et al., 2013, Lu et al., 2014, Lu et al., 2019, Majtan et al., 2014, Halstead et al., 2016). The mechanisms to prevent the formation of biofilm in *P. aeruginosa* and *S. pyogenes* were

examined, and the two species tend to be distinct. In *P. aeruginosa*, fructose plays an important role in preventing biofilm formation by binding to pseudomonas lectin II (LecB) (Lerrer et al., 2007). Additionally, the sugar content of honey was demonstrated to affect the expression of quorum sensing regulatory genes, such as *las* and *rhl* genes, therefore disrupting the quorum sensing system (Wang et al., 2012). However, by exposing *S. pyogenes* biofilm to sub-MIC concentration of Manuka honey, the two adhesive surface protein, Sfo and SfbI, which were related to the initial production of a biofilm, were found to be present at lower than normal levels (Maddocks et al., 2012).

It is important to note that the amount of active honey within a wound dressing and the duration of Manuka honey exposure is crucial for biofilm inhibition instead of stimulation in a wound area. One study found that the maximum inhibition of *P. aeruginosa* biofilm by using 40% (w/v) of Manuka honey (M109 with non-peroxide activity equivalent to 18%w/v phenol) was observed at 11 hours while an increase in biofilm biomass was seen at 24 h indicated that the inhibitory effect was not long-term (Okhiria et al., 2009). Basic sugars like glucose is a natural food source that was used by the bacteria as energy source and building blocks for the formation of biofilm (Stepanović et al., 2007). In addition, a study conducted by Camplin and Maddocks found that isolates of *P. aeruginosa* recovered from Manuka honey treated biofilm exhibited enhanced biofilm-forming capacity when compared to progenitor cells (Camplin and Maddocks, 2014).

1.5.5 Anti-inflammatory properties of honey

Impaired chronic wound healing can occur as a result of deficiencies in the host immune system. The anti-inflammatory properties of honey have been demonstrated in numerous in vitro studies (Henriques et al., 2006, Tonks et al., 2001, Tonks et al., 2003, Leong et al., 2012). Honey is purportedly able to promote wound healing by stimulating the local immune response

(Tonks et al., 2001, Tonks et al., 2003, Gannabathula et al., 2012). Manuka honey stimulates the release of inflammatory cytokines and tumour necrosis factor-alpha (TNF-α) from monocytes, which are known to play a role in wound healing and tissue repair (Tonks et al., 2001, Tonks et al., 2003). A number of clinical trials have recorded decreased symptoms pertaining to inflammation (Efem, 1993, Subrahmanyam, 1998, Subrahmanyam, 1993). Honey's anti-inflammatory activity also showed reduced wound exudate and promotion of granulation tissue formation and epithelization (Ahmed et al., 2003, Efem, 1993, Dunford et al., 2000). Additionally, animal studies have supported these clinical findings and revealed that the application of honey leads to a reduction in inflammation relative to specific non-treated controls (Oryan and Zaker, 1998, Almasaudi et al., 2016).

Despite these findings there is limited information with regards to the exact mechanism for the anti-inflammatory action of honey. Previously it has been proposed that honey inhibits the synthesis of prostaglandin which is mainly responsible for the observed signs of inflammation such as heat, itchiness, and pain (Kassim et al., 2010). Hydrogen peroxide and major royal jelly protein (MRJP) are reported as the major components of honey that mediate these anti-inflammatory effects (Burdon, 1995, Bang et al., 2003, Hadagali and Chua, 2014). Hydrogen peroxide is secreted as a part of the normal inflammatory response to injury and as a by-product of the Maillard reaction in honey which acts as an antiseptic, stimulating the growth of epithelial cells and fibroblasts as well as stimulating angiogenesis in the wound tissue (Burdon, 1995, Hadagali and Chua, 2014). MRJP, is also secreted from the hypopharyngeal and mandibular glands of adult worker bees, which promotes the honey's anti-inflammatory action due to its possible anti-radical and anti-oxidative effects (Aslan and Aksoy, 2015).

1.5.6 Methods to assess the antibacterial activity of honey

Many studies showing the antibacterial activity of honey have been published and it is purported to inhibit above 80 microbial species (Cooper et al., 2002, Cooper et al., 1999, Johnson et al., 2005). However, the various methodologies and the range of samples examined provide contradictory data of the susceptibility of particular microorganisms (Cooper, 2007). Currently, the most commonly used method for estimating the antibacterial activity of medical-grade honey is the agar well diffusion assay against *S. aureus* (Sherlock et al., 2010, Mavric et al., 2008, Lin et al., 2009). It is also known as the phenol equivalence test. This agar diffusion assay works by preparing a growth medium plate, inoculated with a microbial culture, and application of a honey solution to a small area to generate an observable zone of growth inhibition (Allen et al., 1991). However, many drawbacks of the agar diffusion method have been noted previously by authors. These includes insensitivity, where low levels of antimicrobial activity are not generally measurable (Allen et al., 1991) and variability, where small differences in experimental conditions such as agar type and depth, inoculum concentration and incubation conditions can lead to substantial variability in results (Boorn et al., 2010). For example, the antimicrobial activity of T stingless bee honey has been documented in two research papers. Both used the agar diffusion assay, however, one registered no antimicrobial activity (Kimoto-Nira and Amano, 2008), while the other showed significant activity (Irish et al., 2008). Even though variation in the results obtained could be due to the different stingless bee honeys tested, the differences in protocols between researchers is likely to also be a factor (Boorn et al., 2010). Therefore, a standardised approach akin to what we see with the EUCAST antibiotic disc diffusion methodology is needed.

The broth microdilution assay is commonly used in the determination of antimicrobial susceptibility. The main advantages of this method that it is quantitative in approach and actually gives the concentration of honey rather than a series of zone sizes in mm (Kwakman

and Zaat, 2012). Determination of MIC in broth is typically considered a more sensitive and quantitatively reliable approach for studying antimicrobial activity compared to an agar-well diffusion test as the diffusion rate of active compounds in broth may be faster than in agar (Tan et al., 2009, Okeke et al., 2001). However, it is important to note that the lack of uniformity in data and methods use to performed different research studies is one of the drawbacks associated with conducting research on natural products.

1.6 Acquired and adaptive bacterial resistance

Bacteria develop resistance to antimicrobials through four main mechanisms, including 1) reduced uptake of drug; 2) inactivation of the drug; 3) modification of the drug target and; 4) active efflux (Reygaert, 2018). In broad terms, bacterial resistance can be characterised as intrinsic, acquired, or adaptive. Intrinsic resistance can be defined as a trait that is universally shared within a bacterial species. It is not associated with horizontal gene transfer or to previous antibiotic exposure (Martinez, 2014, Cox and Wright, 2013). Decreased permeability of vancomycin across the outer membrane of Gram-negative organisms is an example of intrinsic bacterial resistance (Fernández and Hancock, 2012, Fernandes et al., 2017). Acquired resistance occurs when a previously susceptible bacterium develops resistance to an agent following the acquisition of new genetic material (plasmid, transposons, integrons or DNA) via horizontal gene transfer. The spread of plasmids encoding beta-lactamase genes that permit an organism to hydrolytically cleave members of the beta-lactam class of antibiotics is an example of an acquired resistance trait (Blair et al., 2015, Olivares Pacheco et al., 2013).

Adaptive resistance refers to a transient increase in a bacteria's ability to withstand an antimicrobial as a consequence of changes in gene and/or protein expression. Such observations may occur following exposure to an environmental stimulus, such as exposure to

sub-inhibitory levels of an antimicrobial. Adaptive resistance is often characterized by a transient nature and frequently reverts back to the baseline following elimination of the stimulus (Fernández and Hancock, 2012). Studies that have been carried out on adaptive resistance have suggested that the process requires epigenetic inheritance and heterogenicity of gene expression patterns in bacteria (Motta et al., 2015, Erickson et al., 2015). These expression patterns are associated with increased expression of efflux pumps or reduced entry channels, like porins (Motta et al., 2015). Environmental changes can also result in unstable (transient) adaptations among bacteria, such as decreased susceptibility to antimicrobial drugs concomitant to a reduction in bacterial fitness (Forbes et al., 2014, Forbes et al., 2015). The bacterial adaptation may be permanent when the environmental changes persist, although the underlying mechanisms by which adaptive resistance occurs and the processes that lead to its reversibility are unclear (Salimiyan Rizi and Noghondar, 2018).

Numerous studies have shown that bacterial exposure to sub-lethal concentrations of antimicrobials can produce strains with transient or sustained reductions in susceptibility to biocides and antibiotics (Forbes et al., 2014, Moore et al., 2008, Latimer et al., 2012, Henly et al., 2019). A study conducted by Braoudaki and Hilton (2004) showed distinct degrees of bacterial resistance to biocides in *E.coli* O157 strains that led to cross-resistance against some clinically relevant antibiotics, including erythromycin, imipenem, tetracycline and trimethoprim (Braoudaki and Hilton, 2004). It is unknown which mechanisms are contributing to the adaptive resistance observed in this study, however, the proposed mechanism is the activation of the efflux pump AcrAB (Braoudaki and Hilton, 2004). Despite this, there is limited evidence to suggest that resistance may occur in clinical situations (Russell, 2003, Forbes et al., 2014, Joynson et al., 2002, Loughlin et al., 2002).

Although the previous literature lacks evidence supporting widespread silver resistance, the excessive use of silver in wound care may be associated with concerns about the possibility of

the promotion of resistance to silver and other antibiotics (Ip et al., 2006, Finley et al., 2015a). Various Gram-negative bacteria were passaged repeatedly either in broth containing a sub-inhibitory concentration of ionic silver or on agar containing > 4mg/l ionic silver for 42 days. No significant reduction in silver susceptibility in all tested bacteria was observed except in one strain of *E. coli* which showed resistance to silver (MIC of AgNO3>256 mg/l) after 6 days. Two point mutations associated with loss of certain outer membrane porins (Omp C/F) and activation of the CusS efflux pump that transport silver out of the bacterial cell created this silver resistance (Randall et al., 2015). However, while this research showed silver adaptation, the overall virulence was not studied, and the data were limited to changes in the silver sensitivity of the planktonic bacteria only (MIC) rather than the biofilm type of bacteria (MBEC).

1.6.1 Efflux pumps

Efflux pumps are energy-dependent systems that have evolved by bacteria to prevent intracellular accumulation of harmful compounds. They play an important role in both intrinsic and acquired antimicrobial resistance mechanisms in Gram negative as well as Gram positive bacteria (Fernández and Hancock, 2012). Essentially, genomic analysis has shown that efflux pumps represent approximately 6-18 % of all transporters in any tested bacterial species (Paulsen et al., 1998). The capability of efflux pumps to be activated by a variety of substances and to eliminate a wide range of structurally unrelated molecules indicates that they may have developed to protect bacteria against natural toxicants in the environment (Marquez, 2005). As a consequence of bacterial exposure to antimicrobial agents, induced expression of efflux pumps may lead to a reduction in susceptibility to the antimicrobial to which it is exposed and potentially to other drugs through the expression of multidrug efflux systems (Chuanchuen et al., 2001, Fernández and Hancock, 2012).

Efflux pumps can be divided into five main families based on the source of the energy required for the transport and the homology in the primary and secondary structures. These include (i) ATP-binding cassette (ABC) transporters; (ii) the major facilitator superfamily (MFS); (iii) the small multidrug resistance (SMR) family; (iv) the multidrug and toxic compounds extrusion (MATE) and (v) the resistance-nodulation-cell division (RND) superfamily. All these transporters utilize proton motive force as a source of energy except the ABC-type systems which use the energy derived from ATP hydrolysis. Detailed information regarding these transporters has been reviewed previously (Schweizer, 2003, Poole, 2004, Marquez, 2005, Fernández and Hancock, 2012). Figure 1.6 shows some examples of the families of efflux pumps found in Gram-positive and Gram-negative bacteria.

The occurrence of mutation in the regulator genes leads to an increase of expression of efflux pumps which results in a lowered concentration of antimicrobial within the bacterial cytoplasm. Thus, the bacteria can survive longer and in the presence of a higher concentration of antimicrobials (Schweizer, 2003, Fernández and Hancock, 2012, Piddock, 2006). For example, point mutations in the RND transporter of *P. aeruginosa* (MexY) lead to increased resistance to multiple drugs, including aminoglycosides, beta-lactams and fluoroquinolones (Vettoretti et al., 2009). Furthermore, a loss of bacterial fitness and virulence properties frequently occurs concomitant to the over-expression of efflux systems. The overexpression of MexAB and MexCD, the RND transporters of *P. aeruginosa*, have been associated with reduced production of proteases and phenazines, and reduced virulence when compared to the wild-type strain in a worm model (Sánchez et al., 2002).
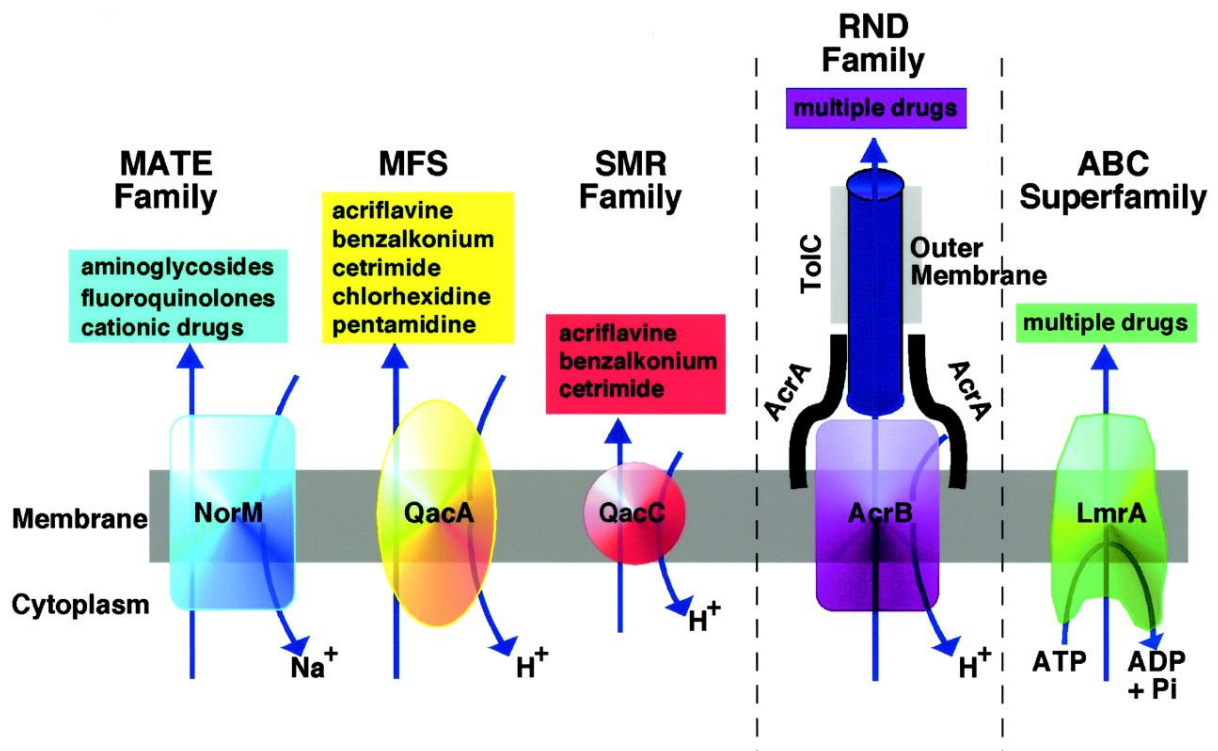
Figure 1. 6 An illustration showing the key members of the five super-families of the efflux pump systems, taken from (Piddock, 2006). NorM; an example of multidrug and toxic compound extrusion family (MATE), QacA; the major facilitator superfamily (MFS), QacC; small multidrug resistance family (SMR), AcrB; resistance-nodulation-cell division (RND), LmrA; ATP-binding cassette (ABC).

1.6.2 Porins

Porins are water-filled pore proteins that extend across the outer membrane of Gram-negative bacteria and mycobacteria. They enable the entry of small hydrophilic compounds, below the threshold sizes of transportable molecules into the cell, that vary from organism to another depending on the diameter of the pores. Porins are classified into two classes that are responsible for the transport of various substances, including the general and selective porins. General porins involved in the general diffusion of the non-specific substance while selective porins are smaller than general porins and allow the absorption of specific substances (e.g. LamB protein for maltose and maltodextrins) (Frenzel et al., 2011, Fernández and Hancock, 2012).

There are many examples of how antimicrobial exposure controls porin expression and the cell envelope's permeability. For example, exposure of *E. coli* bacteria to tetracycline causes downregulation of multiple porins (Lin et al., 2010, Zhang et al., 2008). Also, a reduction in the number of porins such as OmpA, OmpF, and OmpT in *E. coli* was observed after exposure to benzalkonium chloride biocide. Such adaptation conferred increased resistance to this biocide compound as well as to different classes of antibiotics like chloramphenicol, ciprofloxacin, nalidixic acid and ampicillin (Bore et al., 2007).

Additionally, porin-related mutations can affect the expression and/or function of porins which have an impact on the sensitivity of bacteria to antimicrobials. Such mutations may have various effects, such as loss of pore, a change in the size or conductivity of the porin channel, or a reduction of pore expression (Fernández and Hancock, 2012). For instance, a point mutation in OprD in *P. aeruginosa* leads to loss of porin functions and subsequently emergence of carbapenem-resistant clinical isolates (Sanbongi et al., 2009, Wolter et al., 2004).

1.7 Cross-resistance

Bacterial exposure to sub-inhibitory concentrations of antimicrobial may induce a stress response which can lead to changes in cell membrane permeability or activate efflux (Campanac et al., 2002, Walsh et al., 2003). The phenomenon of cross-resistance has been observed in several previous investigations (Walsh et al., 2003, Forbes et al., 2016, Henly et al., 2019). A redaction in ciprofloxacin susceptibility in *S. aureus* and *E.coli* domestic kitchen drain isolate together with a reduction in ampicillin susceptibility in *S. aureus* and *Cronobacter sakazakii* were previously observed after repeated triclosan exposure. Additionally, *S. aureus* showed a significant decrease in susceptibility to ampicillin and ciprofloxacin after repeated exposure to chlorhexidine and polyhexamethylene biguanide (Forbes et al., 2016). Cross-

resistance to ciprofloxacin with ≥7-fold reduction in susceptibility was observed in *E. coli* after exposure to tetracycline. Reduction or loss of OmpF porin and increased efflux pump are believed to be accountable for the induction of ciprofloxacin cross-resistance (Cohen et al., 1989). Also, adaptive resistance to Benzalkonium chloride that causes cross-resistance to ciprofloxacin has been demonstrated in *P. aeruginosa* (Pagedar et al., 2011). Cross-resistance between triclosan and a variety of antibiotics has been reported in *P. aeruginosa*, *S. aureus* and *E.coli* after repeated triclosan exposure (Chuanchuen et al., 2001, Forbes et al., 2016).

1.8 Bacterial resistance to honey

In contrast to antibiotics, non-antibiotic antimicrobials typically exert their effects through interaction with multiple bacterial target sites which makes them less likely to rapidly develop resistance to such agents (Cooper and Gray, 2012). In the laboratory, the repeated passage of bacteria has been shown to result in changes in sensitivity to the tested antimicrobial (Forbes et al., 2014, Forbes et al., 2015, Gilbert and McBain, 2003). Such observations may be relevant with regards to honey. The increased use of honey may also promote honey resistance and may provoke cross-resistance to other antimicrobials (Blair et al., 2009). Honey contains polyphenolic compounds that share target sites with some antibiotics, such as DNA gyrase, in theory, it can be able to induce resistance in similar mechanisms to other antimicrobial agents, such as increased efflux, direct degradation, decreased cell permeability or enhanced detoxification (Adams et al., 2008a, Mavric et al., 2008, Blair et al., 2009, Nolan et al., 2019).

Regarding honey, temporary increases in MIC to Manuka honey have been observed in adapted strains of *E. coli*, *P. aeruginosa* and *S. epidermidis* as reported by Cooper et al. 2010. Here, passaged bacteria were generated through continuous and stepwise training experiments following short-term (10 days) and long-term (28 days) exposure to sub-lethal concentrations

of Manuka honey (Cooper et al., 2010a). Moreover, Ayub and colleagues have investigated the impact of subinhibitory concentrations of branded honey (Marhaba) and unbranded honey (extracted from the *Ziziphus mauritiana* plant), locally available in Pakistan, on *Escherichia coli*, *Salmonella typhi* and *Klebsiella pneumoniae*. They found that continuous exposure to both honeys did not result in the development of any self or cross-resistance to antibiotics (gentamicin, kanamycin and imipenem) in tested bacteria, but the emergence of adapted bacteria capable of growth in adverse physiological conditions was reported (pH 2.5, temperature 60 ∘C) (Ayub et al., 2020). A recent study has reported moderate increases in resistance (maximum two-fold increase in IC_{90}) towards two types of Manuka honey (manuka honey MGO 550$^+$ and Medihoney antibacterial medical honey) after single-step screening assay and serial transfer at increasing concentrations to isolate honey-resistant mutants of *E. coli*. Interestingly, genomic sequencing and experiments on single gene knockouts revealed mutations in *nemAR* and *clpP* genes involved in detoxifying methylglyoxal (Bischofberger et al., 2020). Methylglyoxal (MG) synthesis contributes to the antibacterial function of the *Leptospermum* honeys, so by mutating the genes involved in MG synthesis, the bacteria were able to increase their resistance to honey.

In biofilms, *P. aeruginosa* isolates exposed to sublethal concentrations of Manuka honey have also been shown to develop changes in sensitivity to Manuka honey in conjunction with reduced sensitivity towards rifampicin and imipenem. Whilst no conclusive study has been performed to determine the mechanism of action for such changes, the authors hypothesised that the recovered isolates, which were slower growing than the original strains, were small colony variants (SCV) within the biofilm microbial population (Camplin and Maddocks, 2014). The biofilm mode of growth can exacerbate the problem of antimicrobial resistance by preventing diffusion, where the bacteria may be exposed to sub-lethal antimicrobial concentration. Therefore, the risk of the emergence of bacterial strains with increased tolerance

to antimicrobial agents, as an adaptive mechanism, can be increased (Camplin and Maddocks, 2014). There remain limited studies to date regarding investigating the impact of repeated exposure of bacteria to sub-inhibitory concentration of Manuka honey on bacterial susceptibility and bacterial fitness in planktonic as well as in biofilm status.

While the effectiveness of clinically used antibiotics is waning, one of the therapeutic options is the development of new molecules capable of circumventing bacterial resistance (Wright, 2012, Wright and Poinar, 2012). In the prospective studies to combat key multidrug-resistant pathogens, natural products described as antibacterial have gained a significant position (Langeveld et al., 2014, Khameneh et al., 2019, Ng et al., 2021). However, limited studies have focused on the impact of the continuous bacterial exposure to natural compounds and the subsequent changes in antibiotic sensitivity whereas resistance development during antibiotic therapy was largely reported (Radulovic et al., 2013, Wellington et al., 2013). One study investigated the effect of repeated exposure to *Thymus maroccanus* essential oil and its major component (Carvacrol and thymol) on bacterial susceptibility to different antibiotics groups. Two strains of *E. coli* were cultivated with increasing concentrations of this essential oil and its components, then the susceptibility to various antibiotics was determined by the broth microdilution methods. They found that the adapted strains exhibited a significant increase in the MIC to all tested antibiotics (chloramphenicol, nalidixic acid, erythromycin, and tetracycline) in comparison to the parent strains. This increase in antibiotic resistance correlates with changes in expression of transporters involved in the influx (porins) and the efflux (A crAB family) of different drugs, including natural compounds and different families of antibiotics (Fadli et al., 2014). This kind of bacterial adaptation to natural compounds, such as honeys and essential oils, may occur *in vivo* which makes the bacteria less susceptible to commonly used antibiotics. Therefore, the indiscriminate use of honeys in wound care needs to be carefully monitored.

1.9 Aims and hypothesis of this doctoral project

This doctoral project aims to investigate the effects of repeated sub-inhibitory exposure of a panel of wound isolates to a medical-grade Manuka honey gel in both planktonic and biofilm growth modes. The MIC, MBC and MBEC of Manuka honey and antibiotics used clinically in wound care were determined before (P0) and after ten passages (P10) with sub-lethal doses of Manuka honey and then after ten subcultures of the bacteria in antimicrobial-free media (X10) using an agar-based well diffusion method. Any bacteria showing changes in honey or antibiotic susceptibility were further investigated for changes in virulence. In biofilm exposed cultures, bacterial changes in antimicrobial sensitivity or phenotype were investigated using genomic approaches to better understand the underpinning mechanisms of action. Also, in this doctoral project we studied the hypothesis that repeated exposure of chronic wound isolates to Manuka honey wound gel can lead to changes in antimicrobial sensitivities and virulence.

Chapter 2

General Experimental Methods

2.1 Growth media and Sterilization

All bacteriological media used were purchased from Oxoid (Basingstoke, UK) and prepared by following the manufacturer's instructions. Mueller-Hinton blood agar pre-poured plates (supplemented with 5% w/v horse blood) were purchased from VWR International (Leicestershire, UK). Glassware, disposable pipette tips and media were sterilized before use by autoclaving at 121°C for 15 minutes.

2.2 Antimicrobials

Medihoney antibacterial wound gel (20g tube, Derma Sciences company, Berkshire, UK) was diluted in sterile distilled water to achieve the required concentration (75% w/v). All antibiotics (vancomycin, tetracycline, erythromycin, ciprofloxacin, clindamycin, ampicillin, fusidic acid, gentamicin, meropenem, ceftazidime and cefotaxime) used in this study were purchased from Sigma-Aldrich (Dorset, UK) except for ciprofloxacin, which was obtained from Alfa Aesar (Lancashire, UK). Stock solutions (4000 mg/l) of antibiotics were prepared by dissolving in sterile distilled water and were sterilised by syringe filtration (0.22 µM, Millipore, Watford, UK). Antibiotic-impregnated discs (tetracycline 30 µg, erythromycin 15 µg, ciprofloxacin 5 µg and gentamicin 10 µg) used in the disc diffusion methodology were purchased from Thermo Fisher Scientific Oxoid (Basingstoke, UK).

2.3 Bacterial cultures

Clinical wound isolates were previously isolated from diabetic foot wounds as part of a previous study (Oates et al., 2014). Bacterial panels comprised *Staphylococcus aureus* WIBG 1.2, *Staphylococcus aureus* WIBG 1.6, *Streptococcus pyogenes* WIBG 2.1, *Pseudomonas*

aeruginosa WIBG 1.3, *Pseudomonas aeruginosa* WIBG 2.2 and *Escherichia coli* WIBG 2.4. Methicillin-resistant *Staphylococcus aureus* (MRSA) NCTC 11939 was obtained from the National Collection of Type Cultures (Public Health England). *Staphylococcus epidermidis* ATCC 14990 was acquired from the American Type Culture Collection. All bacteria were grown on Mueller-Hinton agar and incubated aerobically at 37°C for 24h, except for *Streptococcus pyogenes* which was grown using Mueller-Hinton blood agar (5% w/v horse blood).

2.4 Identification of bacteria using 16S rRNA Gene Sequencing

2.4.1 DNA extraction

PCR is a process used to amplify a part of DNA producing thousands to millions of DNA copies. DNA isolation is the first step in the PCR procedure. A single pure colony was selected from an overnight agar plate and suspended in 100µL PCR grade water in a sterile microcentrifuge tube. Then, to breakdown bacterial cells and release DNA, all the suspensions were placed into a 100°C water bath for 10 minutes and then centrifuged at 4000 g for 10 minutes to pellet the cell debris. The supernatant (bacterial DNA) was transferred into sterile labelled microcentrifuge tubes for use in PCR.

2.4.2 Amplification

A PCR reaction mix was made comprising 25µl of MyTaq DNA polymerase ready Mix (Bioline Reagents Ltd, London, UK), 1µl of the forward 8 FLP1 primer (5'-GAGTTTGATCCTGGCTCAG-3') and 1µl of the reverse 806R primer (5'-GGACTACCAGGGTATCTAAT-3'), 18 µl PCR water and 5 µl of the DNA template was

placed into a 0.2 ml PCR tube. The mixture was run in a T-Gradient PCR machine (Biometra, Glasgow, UK) for 35 thermal cycles as follows: 94°C (1 min), 53°C (1 min), 72°C (1 min). A final chain elongation step was incorporated comprising 72°C for 15 minutes. In order to purify the DNA, a QlAquick PCR purification kit (Qiagen, West Sussex, UK) was used following the manufacture's protocol:

1. Add 5 volumes of buffer PB to 1 volume of the PCR sample and mix.

2. Place the QlAquick™ spin column in the 2 ml collection tube given.

3. Apply the sample to the QlAquick column and centrifuge for 30-60 second to bind DNA.

4. Discard the flow-through and return the QlAquick column to the same tube.

5. To wash, add 0.75 ml of buffer PE to the QlAquick column and centrifuge for 30-60 second.

6. Discard the flow-through and position the QlAquick column in the same tube, then, centrifuge the column for another 1 min.

7. Place QlAquick column in a clean 1.5 ml microcentrifuge tube, add 50 µl buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7-8.5) to the centre of the QlAquick membrane and centrifuge the column for 1 min in purpose to elute the DNA.

8. If the purified DNA is to be analysed using a gel, mix 1 volume of loading dye to 5 volumes of purified DNA before loading the gel.

2.4.3 Agarose gel electrophoresis

PCR products were verified for purity using agarose gel electrophoresis, with respect to size of DNA in the samples. A 1% agarose gel was prepared by dissolving 0.4 g of molecular grade agarose powder (Bioline Reagents Ltd, London, UK) in 40 ml of Tris-acetate-

ethylenediaminetraacetic acid (TAE) buffer (50 x stock: 40 mM Tris base, 20 mM glacial acetic acid and 1 mM EDTA, pH 8) and 5 µl of each sample was loaded into each well. To determine the band sizes of the samples, a Hyperladder IV (Bioline Reagents Ltd, London, UK) was used as a marker and the samples run for approximately 1 hr at 70v. Bands were visualised using an ultraviolet (UV) T2201 trans-illuminator (312nm wavelength, Sigma, Poole, UK).

2.4.4 Gene Sequencing

The amount of DNA present in each sample was quantified using a Nanodrop Lite spectrophotometer (Thermo Scientific, Wilmington, USA). Pre-mixes of 16S rRNA gene sequencing reactions were prepared in low rise 96-well PCR plates (STARLAB Int, GmbH, Hamburg, Germany) and comprised the following: 40-50 ng 16S rDNA; 4 pmol of the reverse primer (806R) adjusted to a final volume of 10 µl in PCR grade water. The 16S rRNA gene sequencing was performed at the Sanger Sequencing facility, University of Manchester using an Applied Biosystems 3730 DNA analyser and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA). After receiving the nucleotide sequences, the BLAST database (Basic Local Alignment Search Tool) in the NCBI nucleotide collection (blast.ncbi.nlm.nih.gov) was used to identify the isolated bacterial strain identity with a species cut off of 98% to determine species identity or 95% database matching to determine genus.

2.5 Repeated exposure of bacteria to Manuka honey

Bacterial isolates were continually exposed to sub-lethal concentrations of medical-grade manuka honey using an agar-based diffusion assay (Perez, 1990). In brief, wells were formed at the centre of Mueller- Hinton agar plates using a heat sterilised cork borer (15mm diameter).

Each parent strain of microorganism (P0) was distributed radially three times around the central well by using a sterile inoculating loop. Then, 500 µL of Medihoney 75% (w/v) solutions were allocated into the well and the plates were incubated at 37˚C for 48 hrs (Figure 2.1). After incubation, the bacteria that exhibited growth at the innermost edge of the exposure plates were aseptically taken and re-inoculated onto a fresh exposure plate. This procedure was repeated until the tenth passage (P10) was achieved. After that, and to assess any permanent or transient changes in bacterial susceptibility, the bacteria were sub-cultured in honey-free media for further subcultures to create strain X10. All isolates at P0, P10, and X10 were archived at -80˚C in 50% glycerol for further phenotypic and genomic testing.

Figure 2. 1 Agar-based diffusion assay.

2.6 Bacterial susceptibility testing

2.6.1 Minimum inhibitory concentrations (MICs)

MICs were defined as the lowest concentration of an antimicrobial that inhibits visible microbial growth after overnight incubation. The MICs for antibiotics and manuka honey were assessed using the microdilution method as described previously (Humphreys et al., 2011). Briefly, overnight bacterial cultures were prepared using Mueller-Hinton broth and the suspensions were adjusted to an OD_{600} of 0.8. The cultures were then diluted to 1:100 using double strength Mueller-Hinton broth and aliquots (150 µL) were dispersed in a 96 well microtiter plate. Dilutions of manuka honey wound gel that varied by 5% (w/v) intervals or doubling dilutions of relevant antibiotics (stock solution 4000mg/l) were prepared ordinally across the plate from stock solutions. Positive (inoculated broth without antibiotic) and negative (uninoculated broth) controls were added to the plate and incubated at 37˚C for one day. By visually comparing the turbid wells to the negative control, bacterial growth was confirmed during the procedure. Each bacterium was tested in triplicate, along with a biological replicate.

2.6.2 Minimum bactericidal concentrations (MBCs)

To establish the minimum bactericidal concentration (MBC), aliquots of 10 µL were taken from the wells with no turbidity and spotted onto the surface of fresh Mueller Hinton agar plates in triplicate (2 biological with three technical replicates each). Then, the plate was incubated overnight at 37˚C before reading the results. The lowest antibiotic concentration that resulted in prevention of bacterial growth was recorded as the minimum bactericidal concentration (MBC).

2.6.3 Minimum bactericidal eradication concentrations (MBECs)

MBECs were determined using MBEC assay^{TM} plate (Ceri et al., 1999). Briefly, overnight bacterial cultures were grown, adjusted to an OD_{600} of 0.8, then, further diluted 1:100 into 20 ml of Mueller Hinton broth per plate. Therefore, 100μL of bacterial inoculum was transferred into each well of the MBEC assay^{TM} plate and incubated at 37˚C for 48-72 h to support biofilm formation. Following this, transportable pegged lids were rinsed twice (10 seconds each) in sterile phosphate-buffered saline (PBS) and transferred to an antimicrobial challenge plate containing doubling dilutions of the applicable antibiotic. The plate was incubated for 1 day at 37 ˚C. After that, the pegged lid was moved to a recovery plate that contained 200μL of sterile broth, sonicated on high (approximately 50 kHz for 5 mins) using a SC-52TH Sonicator (Sonicor, New York, USA) to detach sessile cells and incubated at 37˚C for 24 h. The minimum biofilm eradication concentrations (MBECs) were established as the lowest concentration of antibiotic that necessary to eliminate already formed biofilm.

2.7 Planktonic growth rate

All bacterial isolates (P0, P10 and X10) were grown in Mueller Hinton broth overnight. Broth cultures were adjusted to an OD_{600} of 0.8, further diluted 1:100 in MHB (5 ml) and deposited into 96 well plates in triplicate. The culture plate was placed into a microplate spectrophotometer (PowerWave^{TM} XS, BioTek, Swindon, UK). To determine the planktonic growth rate of bacteria, the culture plate was read every hour for 24 h using Gen5^{TM}1.08 software (BioTek, Bedfordshire, UK).

Chapter 3

Exposure to a Manuka honey wound gel is associated with changes in bacterial antimicrobial susceptibility

Abstract

Background. The use of Manuka honey for the topical treatment of wounds has increased worldwide owing to its broad spectrum of activity towards bacteria in both planktonic and biofilm growth modes. Despite this, the potential consequences of the bacterial exposure to Manuka honey, as may occur during the treatment of chronic wounds, are not fully understood. **Methods.** An agar diffusion method was used to repeatedly expose 8 wound isolates, including *Staphylococcus aureus* (n=2), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* (n=2) and *Escherichia coli*, to sub-therapeutic concentrations of a Manuka honey-based wound gel to over ten passages (P10) and again following ten additional passages in honey-free media (X10). Antibiotic sensitivity testing was performed using broth microdilution and disc diffusion methodologies. MIC, MBC and MBEC were determined for each parent strain (P0) and Manuka honey adapted strains (P10 and X10). **Results.** Changes in antibiotic sensitivity above 4-fold were predominantly related to increased vancomycin sensitivity in the staphylococci. Interestingly, *S. epidermidis* displayed phenotypic resistance to erythromycin following passaging, with susceptibility profiles returning to baseline in the absence of further honey exposure. In general, changes in susceptibility to the tested wound gel were moderate ($\leq$ 1-fold) when compared to the respective parent strain. In sessile communities, increased biofilm eradication concentrations over 4-fold occurred in a wound isolate of *Pseudomonas aeruginosa* (WIBG 2.2) as evidenced by a 7-fold reduction in gentamicin sensitivity following passaging. **Conclusion.** Passaging in the presence of a Manuka honey wound gel led to changes in antimicrobial sensitivity that varied between test bacteria. Further investigation is warranted with regards to the effects of Manuka honey passaging on virulence.

3.1 Introduction

Chronic wounds, such as diabetic foot ulcers, are associated with increased morbidity and mortality worldwide (Majtan et al., 2014). A wound is usually considered chronic if it has failed to heal within 8 weeks and leads to significant tissue loss as a result of disruption during the wound healing stages (McCarty et al., 2012). The impairment of wound healing is caused by a range of factors, with bacterial infection frequently cited as a major contributor and aggressive treatment is usually required (Edwards and Harding, 2004, Healy and Freedman, 2006, Lu et al., 2014). The microbiology of chronic wounds is complex and incompletely understood, although studies aiming to profile venous leg ulcers have identified both *S. aureus* and *P. aeruginosa* in over 90 and 50% of samples, respectively (Davies et al., 2007, Han et al., 2011). Other taxa have also been reported, including *Enterococcus faecalis*, coagulase-negative staphylococci, *Streptococcus* spp., members of the *Enterobacteriaceae* and anaerobic rods (Han et al., 2011, Oates et al., 2012b). An increase in the number of antibiotic-resistant bacteria is a cause for concern in wound management and effective control must be accomplished (Bradshaw, 2011).

Honey has been reported to contain over 200 compounds, including sugar, vitamins, amino acids, minerals, enzymes, flavonoids, antioxidants, and phenolic acids (Eteraf-Oskouei and Najafi, 2013, Schneider et al., 2013, Alvarez-Suarez et al., 2014a, Stephens et al., 2015). Manuka honey (derived from the *Leptospermum scoparium* tree in New Zealand) is frequently applied in the treatment of bacterial infections (Qamar et al., 2017) and exhibits well documented antibacterial properties as a result of various phenolic compounds (Carter et al., 2016a, Johnston et al., 2018) and methylglyoxal, the latter following inhibition of bacterial DNA and protein synthesis (Jervis-Bardy et al., 2011, Kilty et al., 2011, Hayes et al., 2018). At bactericidal concentrations, Manuka honey has been reported to cause loss of membrane integrity in both Gram positive and negative bacteria, including *P. aeruginosa* (Roberts et al.,

2012b). At subinhibitory concentrations, Manuka honey has been shown to inhibit septa formation in the staphylococci (Henriques et al., 2010, Lu et al., 2013) and down regulation of flagella associated genes in pseudomonads (Roberts et al., 2014). This purported broad spectrum of activity offers some utility in the management of chronic wound infections.

Concerns have been raised regarding co-selection for antibiotic resistance among bacteria exposed to non-antibiotic antimicrobial agents (McBain and Gilbert, 2001, Buffet-Bataillon et al., 2012, Wales and Davies, 2015). For example, laboratory exposure to some disinfectants has been shown to induce bacterial adaptations that may result in decreased susceptibility to one or more antibiotics (Chuanchuen et al., 2001, Forbes et al., 2014, Forbes et al., 2015). When the antimicrobial agent is removed, these strains can maintain or lose their insusceptibility. However, there is no evidence to date that this adaptive insusceptibility can occur in clinical practice (Russell, 2003, Forbes et al., 2014). The effect of sub-lethal exposure to Manuka honey has received relatively little research attention although stepwise training experiments using planktonic cultures of *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis* suggest only transient reductions in sensitivity to honey (Blair et al., 2009, Cooper et al., 2010a). The anti-biofilm potential of Manuka honey has been described (Maddocks et al., 2012, Lu et al., 2019, Roberts et al., 2019), although elevations in imipenem MIC of up to 4-fold and increased biofilm-forming potential have been observed in cultures derived from honey exposed sessile communities (Camplin and Maddocks, 2014).

To date, research has investigated the result of honey adaptation on limited bacterial strains and antibiotics. As such, this chapter investigates the consequences of bacterial passage in the presence of a Manuka honey wound gel on antimicrobial susceptibility profiling of various Gram-positive and Gram-negative wound isolates. Also, any permanent or transient susceptibility changes in bacteria towards Manuka honey or selected antibiotics will be assessed using broth microdilution.

3.2 Materials and methods

3.2.1 Growth media and chemical reagents

Unless otherwise stated, all bacteria were grown using Mueller-Hinton (MH) agar and broth, except for *Streptococcus pyogenes* which was grown using Mueller-Hinton blood agar (5% defibrinated horse blood). All culture media were incubated at 37°C for 24h. All dehydrated culture media were purchased from Oxoid (Basingstoke, UK) and sterilized by autoclaving at 121°C and 15 psi for 15 min before use.

Medihoney antibacterial wound gel was obtained from Derma sciences (Berkshire, UK) and stock solutions of 75% (w/v) freshly prepared in sterile distilled water before use. All antibiotic powders (clindamycin, tetracycline, gentamicin, vancomycin, erythromycin, fusidic acid, imipenem and meropenem) were purchased from Sigma-Aldrich (Dorset, UK) except ciprofloxacin which was purchased from Alpha Aesar (Lancashire, UK). Stock solutions of each antibiotic were freshly prepared on the day of sensitivity testing, solubilized in distilled water (4000 mg/l stock) and sterilized by syringe filtration (0.22 µm syringe manufacturer). All antibiotic-impregnated discs (CIP, GEN, TET, ERY) that were used for antibiotic disc diffusion testing were purchased from Oxoid,(Basingstoke, UK).

3.2.2 Bacteria

Wild-type clinical wound isolates including: *S. aureus* WIBG 1.2 and 1.6, *S. pyogenes* WIBG 2.1, *P. aeruginosa* WIBG 1.3 and 2.2 and *E. coli* WIBG 2.4 were previously isolated from diabetic foot wounds as part of a previous study (Oates et al., 2014). Methicillin-resistant *S. aureus* (MRSA) NCTC 11939 was purchased from the National Collection of Type Cultures (Public Health England, UK). *Staphylococcus epidermidis* ATCC 14990 was obtained from the American Type Culture Collection.

3.2.3 Exposure of bacteria to a Manuka honey wound gel

Bacterial isolates were continually exposed to sub-lethal concentrations of Medihoney wound gel by using an agar-based diffusion assay (Perez, 1990). In brief, wells were made using a heat sterilized cork borer (15 mm diameter) at the centre of Mueller-Hinton agar plates. By using a sterile inoculating loop, each parent strain of microorganism (P0) was distributed three times around the central well and 150 µl of Medihoney 75% (w/v) solution was aseptically deposited into the wells. The plates were incubated for 48 h at 37 °C. The zone of inhibition of microorganisms were examined after incubation, and the bacteria showing growth at the innermost edge were removed and transferred to a new passage plate containing honey gel solution. This process was repeated until the tenth passage (P10) was reached. Then, the P10 strain was passaged a further 10 times in honey-free media to generate strain X10 and was used to determine adaptation stability. Bacterial cryo-stocks were prepared for P0, P10 and X10 following mixing of broth cultures with 50% sterile glycerol and storage at -80˚C for further susceptibility testing.

3.2.4 Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

To assess the MICs and MBCs of antibiotics and Manuka honey wound gel against different bacterial strains, the microdilution method was used as described from previous studies (Humphreys et al., 2011). Briefly, overnight microbial cultures were spectrophotometrically adjusted to an OD_{600} of 0.8 and further diluted 1 in 100. For MICs, aliquots of 150µl were transferred to a sterile 96-well microtiter plate containing dilutions of honey wound gel that varied by 5% (w/v) intervals or doubling dilutions of antibiotic (stock solution 4000mg/l) across the plate ordinate. Positive (inoculated broth without antimicrobials) and negative (sterile broth) controls were added to the plate to verify the MIC level after

incubated at 37˚C for 18 h. The MIC was described as the lowest concentration of antimicrobial that prevented visible microbial growth after overnight incubation (Andrews, 2001).

To determine MBCs, 5 µl aliquots were taken from the wells that showed no turbidity, spotted onto MH agar plates, and incubated overnight at 37 ˚C before reading the results. The lowest antimicrobial concentration that showed no growth after incubation was recorded as the MBCs. All sensitivity data were expressed as geometric means following two biological replicates with each comprising three technical replicates.

3.2.5 Minimum bactericidal eradication concentrations (MBECs)

Briefly, overnight microbial broth suspensions were adjusted to an OD_{600} of 0.8 and diluted 1:100 into 20 ml of Mueller Hinton broth. Then, 100 µl of adjusted bacterial suspensions were dispensed into each well of the MBEC assay^{TM} plate (Innovotech, Edmonton, Canada) and incubated for 48 h at 37 ˚C to support biofilm formation. After incubation, the transportable pegged lid was detached, rinsed twice with sterile phosphate-buffered saline (PBS) and transferred to an antimicrobial challenge plate containing doubling dilutions of the tested antibiotic. The plate was incubated at 37 ˚C for 24h after incubation, the pegged lid was moved to a recovery plate containing 200µl of sterile Mueller Hinton broth, sonicated (50 kHz, 5 mins) using a SC-52TH sonicator (Sonicor, New York, USA) to disrupt biofilms from the 96-pegs into the recovery plate and incubated for 24 h at 37 ˚C. The lowest concentration of antibiotic that eradicated the biofilm was reported as the MBEC. The turbidity in wells indicated bacterial growth in comparison to a negative control well.

3.2.6 Disc diffusion assay

Disc diffusion assays were conducted according to the standard method described by the European Committee on antimicrobial susceptibility testing (EUCAST). The following antibiotic discs were used: ciprofloxacin (5μg), gentamicin (10μg), erythromycin (15μg) and tetracycline (30μg). Bacterial suspensions of P0, P10 and X10 bacteria were prepared from overnight agar colonies, suspended in saline and mixed until turbid. Then, the density of the bacterial suspension was adjusted to a McFarland 0.5 standard. A semi confluent lawn was formed by swabbing the adjusted cultures onto the surface of Mueller Hinton agar plates. The antibiotic discs were applied to the surface of the plate and incubated at 37°C. The zone of inhibition was measured after overnight incubation (12h) and compared with EUCAST Clinical breakpoints (www.eucast.org). In addition, the zone of inhibition was compared between P0 (parent strain), P10 (after exposure to honey) and X10 (without honey). The data represent the mean of two biological replicates with each comprising three technical replicates.

3.3 Results

3.3.1 Antimicrobial susceptibility

3.3.1.1 Manuka honey susceptibility

Table 3.1 shows the susceptibility data for all Gram positive and Gram negative bacteria before (P0) and after (P10, X10) passaging in the presence of a Manuka honey wound gel. In general, changes in susceptibility to honey in excess of 2-fold were not noted with regards to MICs and MBCs in all tested strains. The greatest fold-change in MIC was observed between parent and P10 strains of *S. epidermidis* and *P. aeruginosa* WIBG 1.3. However, the increase in MBC values of P10 and X10 strains compared to P0 were only observed in *S. epidermidis*.

A reduction in MICs (≤2-fold) at P10 was observed in *S. aureus* WIBG 1.6 and MRSA. Such reduction remained constant after ten passages with honey free media (X10) only in MRSA.

Table 3. 1 Bacterial sensitivities to a Manuka honey wound gel before and after passaging

Bacterium	MIC (%w/v)			MBC (%w/v)		
	(P0)	**(P10)**	**(X10)**	**(P0)**	**(P10)**	**(X10)**
S. aureus WIBG 1.2	15	15	15	30	30	30
S. aureus WIBG 1.6	15	7.5 (2.7)	15	30	15	30
MRSA	11.66 (2.5)	7.5 (2.7)	7.5 (2.7)	30	30	30
S. epidermidis	15	30	30	30	60	60
S. pyogenes	15	15	15	30	30	30
P. aeruginosa WIBG 1.3	60	70	70	>70	>70	>70
P. aeruginosa WIBG 2.2	30	30	30	>70	>70	>70
E. coli	30	30	30	>70	>70	>70

Data are presented as means from biologically duplicated experiments each comprising technical triplicates. Standard deviations are given in parentheses if the data varied between replicates.

3.3.1.2 Antibiotic susceptibilities

Table 3.2 illustrates the antibiotic susceptibility data of Gram positive bacteria before and after bacterial exposure to honey. Overall, changes in the sensitivity of ≥4-fold to at least one tested antibiotic were noted in all bacteria. All staphylococci showed a ≥ 4-fold increase in sensitivity to vancomycin at P10 and X10. A 6-fold decrease in MBEC in *S. aureus* WIBG 1.6 X10 was also observed with regards to ampicillin. Of note, the susceptibilities of *S. epidermidis* to erythromycin and tetracycline decreased (≥4-fold increase in MIC) after repeated exposure

to Medihoney wound gel (P10). Such changes partially or completely reverted to the baseline level by passage X10. Of note, the increase in MIC observed in *S. epidermidis* to erythromycin was transient but sufficient to cross a clinical breakpoint so that P10 was considered as exhibiting phenotypic resistance.

P. aeruginosa WIBG 2.2 showed a ≥4-fold increase in MIC towards ciprofloxacin at P10, which reverted to pre-exposure levels at X10 (Table 3.3). Additionally, *P. aeruginosa* WIBG 2.2 exhibited ≥4-fold increase in MBEC toward gentamicin at P10, with complete reversion to baseline level at X10. *P. aeruginosa* WIBG 1.3 also exhibited a transient increased (3-fold) in MBEC level toward gentamicin after exposure to Manuka honey.

Table 3. 2 Antibiotic susceptibilities of Gram-positive bacteria before and after treatment with Medihoney

Bacterium	Antibiotic	MIC (mg/l)			MBC (mg/l)			MBEC (mg/l)		
		(P0)	(P10)	(X10)	(P0)	(P10)	(X10)	(P0)	(P10)	(X10)
S. aureus **WIBG 1.2**	Vancomycin	0.98	**0.12**	0.24	15.6	3.90	15.6	62.50	62.50	62.50
	Ciprofloxacin	0.24	0.24	0.24	0.98	0..49	0.98	62.50	31.25	31.25
	Erythromycin	0.49	0.98	0.98	15.6	31.25	15.6			
	Fusidic acid	0.49	0.49	0.49	1.95	3.90	3.90			
	Ampicillin	2000	1000	1000	ns	2000	2000	16000	16000	16000
	Tetracycline	0.98	0.49	0.49	7.81	3.90	3.90			
S. aureus **WIBG 1.6**	Vancomycin	0.98	**0.17 (0.06)**	0.49	0.98	1.95	1.95	125	**31.25**	62.5
	Ciprofloxacin	1.95	0.49	1.95	15.6	3.90	15.6	62.50	62.50	62.50
	Erythromycin	31.25	15.6	15.6	62.5	62.5	62.5			
	Fusidic acid	31.25	15.6	31.25	125	62.5	62.5			
	Ampicillin	2000	1000	1000	2000	2000	2000	16000	4000	**2244(816)**
	Tetracycline	0.24	0.24	0.35 (0.133)	1.95	1.95	1.95			
MRSA	Ciprofloxacin	1.95	1.95	1.95	3.90	3.90	3.90	15.60	15.60	15.60
	Fusidic acid	0.12	0.12	0.12	0.98	1.95	1.95			
	Ampicillin	2000	2000	2000	ns	2000	ns	ns	ns	ns
	Vancomycin	0.98	**0.12**	0.24	3.90	1.95	1.95	15.6	62.50	62.50
S. epidermidis	Ciprofloxacin	0.98	0.49	0.49	1.95	0.98	0.98	7.81	11.03 (4.26)	7.81
	Fusidic acid	0.24	0.24	0.24	1.95	1.95	1.95			
	Vancomycin	1.95	**0.29 (0.12)**	0.98	15.6	3.90	7.81	62.5	31.25	62.5
	Erythromycin	0.49	**15.6**	0.49	1.95	**15.6**	3.90			
	Tetracycline	7.81	**62.5**	15.6	15.6	62.5	31.25			
S. pyogenes	Ciprofloxacin	0.49	0.49	0.49	0.98	0.98	0.98	3.90	3.90	3.90
	Erythromycin	0.24	0.98	0.35 (0.14)	1.95	7.81	1.95			
	Tetracycline	0.24	0.98	0.49	7.81	15.6	7.81			

Bold type indicates a ≥ 4-fold change when comparing baseline sensitivities (P0) to P10 and X10 values. Data are expressed as geometric means from biologically duplicated experiments, with each comprising technical triplicates. Standard deviations are given in the parentheses if the data varied between replicates. Non-susceptible (ns) denotes no sensitivity breakpoint determined as the value was in excess of the antimicrobial concentrations used in the broth dilution

Table 3. 3 Antibiotic susceptibilities of Gram-negative bacteria before and after treatment with Medihoney

Bacterium	Antibiotic	MIC (mg/l)			MBC (mg/l)			MBEC (mg/l)		
		(P0)	(P10)	(X10)	(P0)	(P10)	(X10)	(P0)	(P10)	(X10)
P. aeruginosa WIBG 1.3	Ciprofloxacin	0.29(0.12)	0.24	0.24	0.98	0.49	0.98	7.81	7.81	7.81
	Gentamicin	0.98	3.90	1.95	1.95	7.81	1.95	125	500	125
	Meropenem	0.98	0.98	0.98	1.95	1.95	1.95	31.30	15.6	15.6
P. aeruginosa WIBG 2.2	Ciprofloxacin	0.028	**0.24**	0.028	0.24	0.98	0.24	3.90	3.90	3.90
	Gentamicin	0.98	0.49	0.98	3.90	3.90	3.90	15.6	**125**	15.6
	Meropenem	0.98	0.49	0.49	1.95	0.98	1.95	2.93 (1.129)	3.90	3.90
E. coli	Ciprofloxacin	0.02	0.02	0.02	0.122	0.24	0.24	1	0.5	1
	Gentamicin	0.98	3.90	1.95	3.90	15.6	3.90	250	125	125
	Meropenem	0.12	0.12	0.12	0.24	0.24	0.24	0.98	0.98	0.49

Bold type indicates a $\geq$ 4-fold change when comparing baseline sensitivities (P0) to P10 and X10 values. Data are expressed as geometric means from two biological replicates, with each comprising technical triplicates. Standard deviations are given in the parentheses. Non-susceptible (ns) denotes no sensitivity breakpoint determined as the value was in excess of the antimicrobial concentrations used in the broth dilution.

3.3.1.3 Disc diffusion assay

Bacteria that showed a 4-fold or above difference in MICs and MBCs were further tested using a disc diffusion assay (Table 3.4). *S. epidermidis* exhibited a decrease in sensitivity (above or equal to 2mm difference in inhibition zone size between P0 and P10) toward erythromycin and tetracycline. Interestingly, no zone of inhibition was observed in *S. epidermidis* exposed to erythromycin after 10 passages with Manuka honey wound gel, which support the data of the MIC (Figure 3.1). Both strains of *P. aeruginosa* showed a decrease in the ciprofloxacin zone of inhibition (>3mm) while *P. aeruginosa* WIBG 1.3 showed a 5mm decrease in the zone of inhibition of gentamicin after honey exposure (P10).

Table 3. 4 Antibiotic susceptibility toward bacterial isolates that showed ≥ 4-fold changes in MICs using disc diffusion assay.

	Zone of inhibition (mm)											
	Tetracycline			Erythromycin			Ciprofloxacin			Gentamicin		
Bacteria	P0	P10	X10	P0	P10	X10	P0	P10	X10	P0	P10	X10
S. aureus WIBG 1.6	24.3 (1.8)S	25(S)	24 (0.89)S	16.6 (0.82)R	15.2 (1.17)R	16.2 (0.75)R	20(R)	23(S)	23(S)	-	-	-
S. epidermidis	4(R)	0(R)	3.5 (0.55)R	24(0.89)(**S**)	0(**R**)	23(S)	22(R)	20(R)	20(R)	-	-	-
S. pyogenes	32.6 (0.82)S	28 (0.89)S	30(S)	33.3(0.82)S	30(S)	29.3 (0.82)S	19(S)	19.3 (0.82)S	21(S)	-	-	-
P. aeruginosa WIBG 1.3	-	-	-	-	-	-	37.6 (0.82)S	34.5 (0.55)S	33(S)	24(S)	19 (0.89)S	23.2 (0.75)S
P. aeruginosa WIBG 2.2	-	-	-	-	-	-	38(S)	32.7 (0.82)S	30(S)	22(S)	22(S)	21(S)

Data shows growth inhibition zones (mm) representative of antibiotic susceptibility before and after 10 passages in the presence of Medihoney. Standard deviation is given in parenthesis where data varied around the mean. Data represent two biological and three technical replicates each (n=6). P0= before Medihoney exposure; P10= after 10 passages with medihoney; X10= after passage in the absence of Medihoney https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_11.0_Breakpoint_Tables.pdf
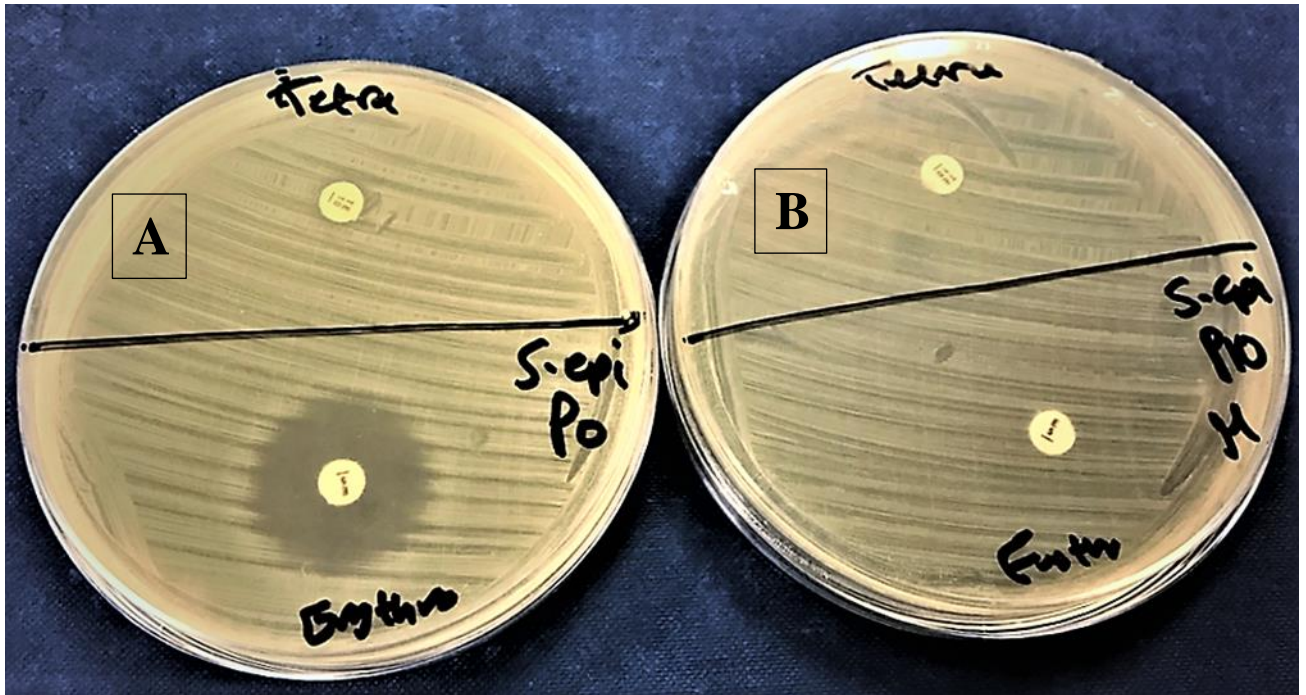
Figure 3. 1 Disc diffusion assay of *S. epidermidis* toward tetracycline and erythromycin.
A) P0; parent strain. B) P10; Medihoney-adapted strain. Of note, no zone of inhibition around
erythromycin disc after Medihoney exposure (P10) and the bacteria changed from
erythromycin-sensitive (24mm) to erythromycin-resistant (0 mm) after Medihoney exposure.

3.4 Discussion

Honey has been used throughout human history for medicinal purposes, with one of the most recent uses being in the form of antimicrobial wound dressings (Carter et al., 2016b). The excessive and inappropriate use of antibiotics has resulted in the emergence of resistant bacteria (Cantón et al., 2013). Modern medicine has continued to struggle with the issue of multi-drug resistant bacteria, which is emerging at a rate greater than the antibacterial treatments manufactured to counter them. However, resistance is not a concept limited to antibiotics (Lin et al., 2011). Other biocides and antiseptics commonly used to treat wounds have also been found to elicit some level of resistance over time and could potentially involve cross-resistance to clinically relevant antibiotics (Braoudaki and Hilton, 2004, Henly et al., 2019, Chuanchuen et al., 2001, Forbes et al., 2015, Walsh et al., 2003, Forbes et al., 2014). For example, Forbes et al, 2016, previously found that repeated exposure to biocides, including chlorhexidine and triclosan, was associated with adaptive changes in bacterial susceptibility to various clinically relevant antibiotics such as ciprofloxacin and tetracycline (Forbes et al., 2016). With regards to honey, such observations have been limited to date although isolates derived from biofilms have shown reduced sensitivity to Manuka honey products as well as reduced sensitivity to rifampicin and imipenem (Camplin and Maddocks, 2014).

In the present study, the exposure of a test panel of bacteria to a wound gel resulted in both increases and decreases in antimicrobial susceptibilities. Overall, changes were relatively moderate (≤ 7.5% w/v) with regards to sensitivity to the Manuka honey wound gel. These observations are in keeping with previous research whereby stepwise resistance training in liquid culture was associated with only minor changes in sensitivity, although observed changes were transient (Cooper et al., 2010b). The clinical significance of these observations is unclear regarding their effect on therapeutic efficacy. The concentrations of Manuka honey included in licensed wound care products are typically over the sensitivities reported in the

present study (Cooper et al., 2010b). Wounds are, however, moist environments that may lead to variable product dilution, as has been discussed by Camplin and Maddocks (Camplin and Maddocks, 2014). Loss of activity following a pH mediated reduction in hydrogen peroxide production may also need to be considered (Bang et al., 2003, Molan and Rhodes, 2015, Cooper, 2016). The low MIC values reported from Manuka honey sensitivity studies have been cited in support of the limited effects that wound dilution is likely to impart upon honey efficacy (Molan and Rhodes, 2015). Such effects are, however, less clear regarding the eradication of the biofilm phenotype from a wound environment.

Cross-resistance remains an area of concern regarding the overuse of antimicrobials (Wales and Davies, 2015). Adaptation to suboptimal antimicrobial exposure may result in alterations in cell wall permeability or efflux systems that negatively impact antibiotic sensitivity profiles. To this end, the exposure of reference strains of *S. aureus* and *P. aeruginosa* to ciprofloxacin, tetracycline and oxacillin have been shown to rapidly generate antibiotic-resistant phenotypes but did not result in observable cross-resistance to honey (Blair et al., 2009). In the present study, whilst resistance to in-use concentrations of Manuka honey were not observed, an additional aim was to investigate the effect of potential honey adaptation on antibiotic susceptibility. Changes in antibiotic profiles were limited, although it must be noted that an increase in MIC occurred in *S. epidermidis* to erythromycin. Whilst these observations were transient (i.e.) the phenotype did not persist in the absence of further wound gel passaging, the increase was sufficient to cross a clinical breakpoint at P10 (The European Committee on Antimicrobial Susceptibility Testing, 2020). The absence of a zone of inhibition in *S. epidermidis* (P10) in the disc diffusion assay was in support of this microdilution data. Trace levels of macrolides have been detected in honey previously (Bargańska et al., 2011), although, such findings are unlikely to be of relevance in formulations utilised in the healthcare setting

given their controlled sourcing and rigorous processing procedures. As such, these data may suggest adaptive resistance via an unknown mechanism.

Changes in antimicrobial sensitivity following honey adaptation in biofilm growth modes have been reported previously (Camplin and Maddocks, 2014). Sessile communities of *P. aeruginosa* were exposed to Medihoney to determine changes in honey inhibitory concentrations, honey biofilm eradication concentrations and antibiotic sensitivities in the residing biofilm biomass. Overall, reduced sensitivities to both imipenem and rifampicin were observed in conjunction with marginal increases in sensitivity in planktonic and sessile honey sensitivities. Biofilms were not directly passaged in the present study but remain of interest as a future research direction. Rather, adapted planktonic cultures were investigated for subsequent changes in MBEC and biofilm-forming potential when tested using a crystal violet assay. Changes in biofilm eradication concentration were marginal in most cases, although a 4-fold reduction in gentamicin sensitivity was observed in a clinical isolate of *P. aeruginosa*. This observation occurred in conjunction with highly significant, but transient, increases in planktonic growth rate and biofilm formation, the later in support of Camplin and Maddocks (2014). In summary, the repeated laboratory exposure of a test panel of bacteria to Manuka honey, in the form of a wound gel, resulted in variable changes in antimicrobial sensitivity when compared to a progenitor. This is an important observation as chronic wounds provide an environment where antimicrobial wound dressings may be present *in situ* over prolonged periods. However, care must be taken in extrapolating the findings of an *in vitro* study to possible clinical effects. It must be noted that planktonic bacteria remained susceptible to concentrations of the Manuka honey wound gel used in clinical application, despite repeated exposures. With the exception of the pseudomonads and gentamicin, changes in biofilm antibiotic sensitivity were favourable. The underlying mechanisms of changes are unclear and warrant further investigation.

Chapter 4

Phenotypic and Virulence Analysis of Bacteria before and after Repeated Exposure to Manuka honey wound gel

Abstract

Background. The laboratory passaging of bacteria to a manuka honey wound gel has been shown to result in modest changes in antimicrobial susceptibility. However, the effect on bacterial phenotype remains unclear. The aim of this chapter is to determine changes in bacterial growth metrics, biofilm formation and virulence following exposure to a commercially available wound gel. **Methods.** Changes in phenotype were determined following the investigation of parent (P0) and passaged bacteria (P10, X10). Test bacteria were as follows: *Staphylococcus aureus* (n=3), *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* (n=2) and *Escherichia coli*. Changes in growth rate metrics were determined following the fitting of absorbance data to the standard form of the logistic equation. Biofilm-forming ability, before and after passage in the presence of manuka honey wound gel, was determined using a crystal violet biofilm assay. Virulence was determined using a *Galleria mellonella* pathogenicity assay, with significant changes in killing to progenitor strains further investigated for changes in motility, haemolysis, pyocyanin, DNase, and coagulase production. **Results.** Following honey wound gel exposure, 4/8 bacteria exhibited enhanced virulence, including *S. aureus* WIBG 1.2, *S. epidermidis*, *P. aeruginosa* WIBG 1.3 and *E. coli*. In the pseudomonads and *S. epidermidis*, this occurred in conjunction with increased haemolysis and biofilm formation, whilst *P. aeruginosa* also exhibited increased pyocyanin production, swimming, and swarming motility. Where virulence attenuation was noted in a passaged wound isolate of *S. aureus* (WIBG 1.6), this was concomitant to delayed coagulation, reduced haemolytic potential and DNase activity. **Conclusion.** Manuka honey wound gel passaging was associated with significant changes in biofilm formation, virulence, and exotoxin production, in the staphylococci and pseudomonads.

4.1 Introduction

Manuka honey is used in modern medicine as a topical antimicrobial agent for wound infections, showing efficacy against a wide variety of pathogens (Cooper et al., 2002). The presence of a multitude of antimicrobial compounds within Manuka honey has been noted, although a significant portion of this antimicrobial effect is attributed to methylglyoxal and high osmotic potential (Roberts et al., 2015). In conjunction with a reported bactericidal effect, several studies have documented the antibiofilm effects of Manuka honey against several pathogens, including pseudomonas and staphylococci biofilms (Lu et al., 2014, Paramasivan et al., 2014, Piotrowski et al., 2017, Maddocks et al., 2013, Lu et al., 2019, Halstead et al., 2016), making it a desirable application in wound care.

Various Manuka honey containing dressings are used for wound management worldwide (Kamaratos et al., 2014, Winter, 2017). These dressings often stay on the skin for extended periods to provide continuous control of microbial growth at the tissue surface. However, the long-term treatment of an infected wound with honey-containing dressings could expose the resident bacteria to sub-therapeutic concentrations of honey as a result of wound dilution by a large volume of exudate or the presence of catalase and/or serum interfering with the activity of some antimicrobial agents in honey, which could theoretically promote bacterial adaptation and resistance to antimicrobials (Amenu, 2013). Previous laboratory exposure to some disinfectants has been shown to induce bacterial adaptations that affect biofilm formation potential (Latimer et al., 2012, Henly et al., 2019), bacterial fitness (Forbes et al., 2015) and pathogenicity (Latimer et al., 2012, Bazaid et al., 2018, Henly et al., 2019). The effect of sublethal exposure to Manuka honey has received relatively little research attention although stepwise training experiments using planktonic cultures of *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis* suggest only transient reductions in sensitivity to honey (Blair et al., 2009, Cooper et al., 2010b).

Although knowledge of acute infection pathogenesis has improved, there has been a lag in understanding of chronic infections. A two-step process can be associated with chronic infection, with different roles mediating initial and developed infection stages. The functions of acute virulence are expected to be active early on, so that bacteria can gain ground in the host. Once the infection is formed, the activity of the virulence factor will decrease, and the formation of biofilms is induced (Smith et al., 2006a, Sousa and Pereira, 2014). Previous studies showed the effect of Manuka honey on various bacterial virulence factors and pathogenicity obtained by proteomic and genomic investigations (Jenkins et al., 2014). A study conducted by Jenkins et al (2014) investigated the effect of 10% (w/v) Manuka honey on the expression of different genes of MRSA. A reduction in expression of agr cluster genes, that regulate many virulence factors including quorum sensing, was observed on MRSA after treated with Manuka honey. In addition, the adhesive genes in *S. pyogenes*, such as *Sof* and *SfbI*, have been found to be down-regulated when treated with Manuka honey (Maddocks et al., 2012). Exposure of *P. aeruginosa* to Manuka honey resulted in a decrease in swimming and swarming motility that was correlated with the reduction of expression of the main structural flagellin protein (FliC) and subsequent suppression of flagellin-associated genes, such as *fliA*, *fliC*, *fleN*, *fleR* and *fleQ* (Roberts et al., 2014). Despite these promising effects, there are concerns about the impact of long-term Manuka honey exposure on bacterial virulence and biofilm formation.

In investigating the effect of any antimicrobial susceptibility changes occurring within the bacteria, bacterial pathogenesis is an essential but often overlooked factor to consider. Passaging experiments that attempt to address such changes have demonstrated that adaptation can potentially manifest in ways other than through changes in drug sensitivity and include changes in biofilm-forming potential, bacterial fitness, and pathogenicity (Latimer et al., 2012, Lu et al., 2014, Henly et al., 2019). These studies have investigated virulence potential using

the *Galleria mellonella* non-mammalian model. When compared to mammalian models, *G. mellonella* wax worms do not require an animal license, ethical approval, or special laboratory equipment to handle, and are cost-effective. Additionally, the wax worm innate immune system broadly represents that of other mammalian models (Cook and McArthur, 2013, Tsai et al., 2016). Whilst the general understanding of such effects in response to Manuka honey are less clear, a handful of gene expression studies support a view of virulence attenuation (Jenkins et al., 2014, Roberts et al., 2014). It must be noted, however, that Manuka honey exhibits a complex mode of action that is capable of acting upon multiple cellular target sites with variable cellular responses to honey reported between different bacterial species (Jenkins et al., 2012, Carter et al., 2016a, Hayes et al., 2018, Johnston et al., 2018).

The data in Chapter 3 demonstrated that continual exposure of a test panel of wound bacteria to Manuka honey wound gel resulted in variable changes in both honey and antibiotic sensitivities when compared to a progenitor. Consequently, this chapter will further identify the additional effects of repeated Manuka honey exposure in wound bacteria by assessing changes in pathogenicity, planktonic growth rate, biofilm formation ability and exotoxins production.

4.2 Materials and methods

4.2.1 Bacteria

Two strains of methicillin-sensitive *Staphylococcus aureus* (WIBG 1.2 and WIBG 1.6), two strains of *Pseudomonas aeruginosa* (WIBG 1.3 and 2.2), *Streptococcus pyogenes* WIBG 2.1, and *Escherichia coli* WIBG 2.4 were used in this study and were originally collected from diabetic foot wounds as part of a previous study (Oates et al., 2014). Methicillin-resistant *Staphylococcus aureus* (MRSA) NCTC 11939 was acquired from the national collection of

type cultures (Public Health England). *Staphylococcus. epidermidis* ATCC 14990 was obtained from the American Type Culture Collection. Parent strains (P0), those passaged ten times on Manuka honey wound gel (P10), and those passaged a further ten times in honey-free media (X10) were previously created using an agar-based diffusion assay (Section 2.6) and archived at -80 °C.

4.2.2 Growth Media

Except for *S. pyogenes,* which was cultured onto Mueller-Hinton agar supplemented with 10% (w/v) defibrinated horse blood (Oxoid Ltd, UK), all bacteria were cultured onto Mueller-Hinton agar. All cultures were incubated aerobically at 37°C for 24 h. All dehydrated bacteriological media were purchased from Oxoid (Basingstoke, UK) and autoclaved at 121°C and 15 psi for 15 min before use. Both DNase test agar and blood supplemented agar used in haemolysin tests were purchased from Oxoid (Basingstoke, UK).

4.2.3 Planktonic growth rate

Overnight cultures of all bacterial isolates (P0, P10, and X10) were adjusted to an OD_{600} of 0.8, further diluted 1:100 in Mueller Hinton broth and deposited into 96 well plates. To determine the planktonic growth rate of bacteria, the culture plate was placed into a microplate reader (PowerWave^{TM} XS, BioTek, Swindon, United Kingdom) and the optical density was read every hour for 24 h using Gen5^{TM} software (Version 1.8; BioTek, Swindon, United Kingdom). Growth curve data from eight absorbance readings (biological duplicates each comprising 4 technical replicates) were fitted to a standard form of the logistic equation using the R software package Growthcurver (Sprouffske and Wagner, 2016) to determine metrics relating to intrinsic growth rates (r, h^{-1}), carrying capacity (K) and maximum generation time

(t_gen; h^{-1}). Pairwise statistical comparisons of generated datasets were performed between parent and passaged mutants (P0 vs. P10; P0 vs X10) at $P \leq 0.05$ using a Wilcoxon signed-rank test. Comparisons were performed using SPSS version 22 (IBM analytics, New York, United States).

4.2.4 Crystal violet biofilm assay

The potential to form biofilms was compared in parent, P10 and X10 bacteria using a crystal violet assay. Overnight bacterial cultures were adjusted to an optical density of 0.8 and then diluted 1:100 in Mueller Hinton broth. Aliquots (150 µl) of diluted bacterial culture were transferred to the wells of a sterile 96-well microtiter plate (Corning Ltd, Weisbaden, Germany) and were incubated aerobically for 48 h at 37 °C. After 48 h, the liquid in the wells was removed by inversion of the microtiter plate, and the wells were washed twice using 200 µl of sterile phosphate-buffered saline (PBS). The wells were stained with 250 µl of 1% (w/v) crystal violet solution for 1 min, rinsed twice with PBS and left to dry at room temperature. To solubilise the attached crystal violet, 300 µl of absolute ethanol was added to each well (10 min) before measuring the absorbance (OD_{600}) using a PowerWave™ XS plate reader (BioTek, Swindon, United Kingdom). Data were presented as biofilm units calculated by dividing the absorbance of the crystal violet bound biofilm by a corresponding planktonic OD_{600} in order to adjust for planktonic mass. All data points were plotted and analysed using GraphPad Prism version 7.0 (GraphPad Software, California, United States) and are presented as means of biologically duplicated experiments, each comprising six technical repeats. Differences between the parent and the passaged bacteria (P0 vs. P10; P0 vs. X10) were determined using a Mann-Whitney test.

4.2.5 *Galleria mellonella* pathogenicity assay

The methodology was performed as described previously (Latimer et al., 2012). Larvae of *Galleria mellonella* were purchased from Live Food Direct (Sheffield, United Kingdom) and stored in the dark for a maximum of 7 days. For each treatment group, 10 larvae were randomly assigned and placed in Petri dishes. Overnight suspensions of P0, P10 and X10 bacteria were centrifuged (3,102 g, 10 min) and washed twice using sterile PBS. Then, bacterial suspensions were adjusted using a light spectrophotometer and corresponding CFUs determined through viable counting. Briefly, quantification was performed following 1 in 10 serial dilutions in Mueller Hinton broth. Dilutions were plated in triplicate onto Mueller Hinton agar and incubated overnight (18 h; 37°C). The corresponding standard bacterial inoculum were as follows: *S. aureus* WIBG 1.2 ($OD_{600}= 0.1$, 1.2×10^9 CFU/ml); *S. aureus* WIBG 1.6 ($OD_{600}= 0.1$, 1.3×10^9 CFU/ml); MRSA ($OD_{600}= 0.1$, 1.6×10^9 CFU/ml); *S. epidermidis* ($OD_{600}= 0.1$, 5.8×10^8 CFU/ml); *S. pyogenes* ($OD_{600} = 0.1$, 1.4×10^8 CFU/ml); *P. aeruginosa* WIBG 1.3 ($OD_{600}= 0.1$ followed by 1:1000000 dilution, 250 CFU/ml), *P. aeruginosa* WIBG 2.2 ($OD_{600}= 0.1$ followed by 1:1000000 dilution, 64 CFU/ml) and *E. coli* ($OD_{600}= 0.1$ followed by 1:500000 dilution, 1.9×10^4 CFU/ml). These dilutions were determined following in-house testing to achieve observable kill rates across the 7 day test period. Each of the larvae was injected with 5µl of adjusted bacterial suspension into the hemocele via the last left proleg using a sterile Hamilton syringe (Sigma, Dorset, United Kingdom). Larvae were incubated in a petri dish at 37 ° and the number of surviving individuals recorded daily for up to 7 days. An untreated group (no injection) and a group injected with sterile PBS were used as a controls. All experiments were performed as biological duplicates with each assay comprising ten worms. Tests were terminated when two or more of the control larvae died. The data were presented as a Kaplan-Meier survival curves and intra-strain, pairwise comparisons of datasets (P0 vs.

P10; P0 vs. X10) were conducted using the log-rank test in Graph Pad Prism 7 (GraphPad Software, California, United States).
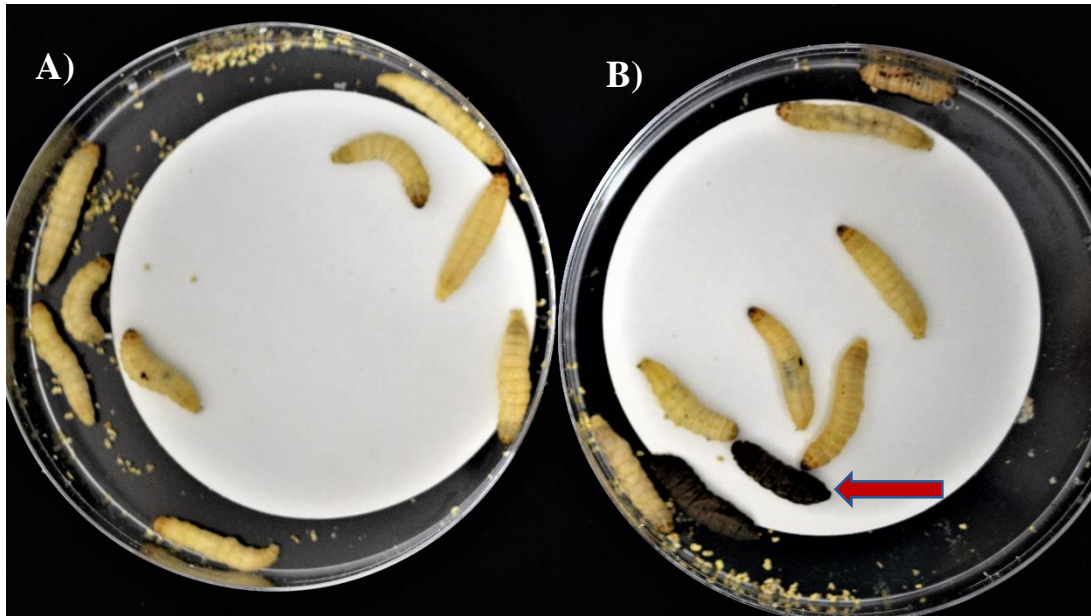
Figure 4. 1. *Galleria mellonella* **pathogenicity assay.** A) Larvae before bacterial injection (creamy white in colour). B) Melanisation of larvae after bacterial injection (black, indicated by red arrow).

4.2.6 Determination of bacterial haemolysin activity

Haemolytic activity was measured for all strains that showed a significant change in pathogenicity and exhibited haemolysis when grown in blood agar (Latimer et al., 2012). P0, P10 and X10 passaged bacteria were grown in Mueller Hinton Broth overnight at 37 °C. The overnight cultures were diluted 1:100 and incubated at 37 °C until an OD_{600} of 0.3 was achieved. Then, whole defibrinated horse blood (5 % v/v; Oxoid Ltd, Basingstoke, United Kingdom) was added to the samples and also to sterile broth (negative control). All assay reactions were incubated in a shaking incubator (100 rpm, 37 °C) for 3 h. 1 ml aliquots were then removed and centrifuged at 16,000 X g for 4 min (1-14 Microfuge, Sigma-Aldrich, Dorset, United Kingdom). Optical density measurements of the supernatant were determined using a

light spectrophotometer (540 nm). To control for variability in growth rates, haemolytic activity was adjusted according to viable counts. To do this, serial dilutions (1 in 10) were performed, plated onto mannitol salt agar, and incubated overnight (18 h; 37 °C). Percentage haemolysis was expressed as the change in A_{540} (ΔA_{540})/cfu. Statistical comparisons (P0 vs. P10; P0 vs. X10) were performed in GraphPad Prism version 7 (GraphPad Software, California, United States) using a student's unpaired T-test with Welch's correction. Data are presented as means from biologically replicated experiments (n= 4).

4.2.7 Determination of bacterial DNase activity

Overnight cultures of *S. aureus* (P0, P10, and X10) were adjusted to an OD_{600} of 0.8, then further diluted 1:100 in Muller-Hinton Broth and incubated at 37 °C to reach an OD_{600} of 0.3. Aliquots (5 µl) were spotted onto the DNase agar surface (Oxoid, UK) and plates were incubated overnight at 37 °C before coating with 1 M hydrochloric acid (Sigma-Aldrich, UK). DNase activity was reported when a zone of clearance was observed around the colony. Data are expressed as means of biologically duplicated experiments, each comprising three technical replicates.

4.2.8 Coagulase assay

Overnight cultures of P0, P10, and X10 *S. aureus* (WIBG 1.2 and 1.6), MRSA and *S. epidermidis* were adjusted to an OD_{600} of 0.4. Aliquots (1 ml) were added to 3 ml of rabbit plasma with EDTA (Bactident coagulase, Merck, Darmstadt, Germany) and incubated at 37 °C in a water bath. Tubes were examined for signs of coagulation over 3 h and scored on a five-point scale according to the manufacturer's instructions. Assays were performed as biological triplicates. *S. epidermidis* ATCC 14990 was included a negative assay control.

4.2.9 Pyocyanin assay

Differences in pyocyanin production between parent, P10 and X10 bacteria were performed using a chloroform extraction method to better understand differences in virulence potential before and after wound gel exposure. Pyocyanin was determined as described elsewhere (Essar et al., 1990). 10-ml of overnight bacterial culture was grown (37°C, 200 rpm for 24 h) in PB medium (20 g Bacto peptone, 1.4 g $MgCL_2$, 10 g K_2SO_4) to maximize pyocyanin production. 6 ml of chloroform was added to 10 ml of cell-free supernatant and shaken vigorously until the pyocyanin was extracted into the chloroform layer. The chloroform layer was drawn off and vigorously mixed with 2 ml of 0.2 N HCL to give a pink to deep red solution. The absorbance of this extracted solution was measured at $OD_{520\ nm.}$ The percentage of pyocyanin production was expressed relative to the parent strain as follows: A_{520} (ΔA_{520})/cfu. Viable counts from corresponding 24 h PB cultures were determined as described for the *Galleria mellonella* assay (section 4.2.5). Pyocyanin production experiments were performed as biological triplicates and the data expressed as means using GraphPad Prism version 7 (GraphPad Software, California, United States). Comparisons between parent and passaged bacteria (P0 vs. P10; P0 vs X10) were determined using an unpaired T-test with Welch's correction.

4.2.10 Motility assay

A motility assay was adapted from a previous, published methodology (Rashid and Kornberg, 2000). The diameters of motility zones in all cases were determined by taking the mean of two perpendicular measurements of swimming, swarming, and twitching motility (two biological and three technical replicates each, n=6).

4.2.10.1 Swimming motility

Briefly, swimming agar plates were prepared using 15 g/L of Luria broth and 3 g/L of nutrient agar and prepared in distilled water. The plates were dried for 15 mins under laminar flow before inoculation with 3 µl of adjusted (OD$_{600}$=0.3) of overnight cultures of *P. aeruginosa* WIBG 1.3 (P0, P10 and X10). Plates were incubated at 37 °C for 24 h before data recording.

4.2.10.2 Swarming motility

Swarming agar plates were prepared from 8 g/L of nutrient broth (Oxoid, UK), 50 ml 10% glucose (Sigma-Aldrich, UK), 5g/L of nutrient agar (Oxoid, UK) and sterile distilled water. Plates were dried in a lamina flow cabinet and inoculated using adjusted pseudomonad cultures as described in section 4.2.10.1.

4.2.10.3 Twitching motility

Twitching plates were prepared using 25 g/l of Luria broth with 10 g/l nutrient agar, then dried under the laminar flow for 1 h before inoculation. The plates were inoculated from an overnight Muller-Hinton agar plate culture of *P. aeruginosa* (P0, P10 and X10) by stabbing through the agar to the bottom of the agar plate with a sterile toothpick. Plates were incubated for 24 h at 37 °C.

4.3 Results

4.3.1 Modelling of growth curve data

For the eight strains examined for alterations in growth curve metrics, 5/8 showed significant changes in either carrying capacity, intrinsic growth rate or generation time after passage exposure to Manuka honey wound gel (Table 4.1). *S. aureus* WIBG 1.6, *S. aureus* WIBG 1.2 and *E. coli* exhibited a significant increase in both carrying capacity and doubling

time with a significant decrease in intrinsic growth rate in both P10 and X10 bacteria. Additionally, *S. epidermidis* showed an increased doubling time concomitant to a reduction in intrinsic growth rate at X10. In contrast, *P. aeruginosa* WIBG 1.3 exhibited a significant and sustained decrease in both carrying capacity and doubling time following passaging.

Table 4. 1 Growth curve metrics for parent and passaged bacteria.

Bacterium	Carrying capacity (k)			Growth rate (h^{-1})			Doubling time (h^{-1})		
	P0	P10	X10	P0	P10	X10	P0	P10	X10
E. coli	0.37 (0.07)	0.35 (0.03)	0.40 (0.04)	0.52 (0.07)	0.35 (0.02) **	0.37 (0.03) **	1.25 (0.16)	1.97 (0.10) **	1.78 (0.36) **
P. aeruginosa WIBG 1.3	0.52 (0.10)	0.30 (0.02) **	0.32 (0.03) **	0.54 (0.21)	1.44 (0.20) **	1.60 (0.36) **	1.43 (0.42)	0.49 (0.07) **	0.45 (0.10) **
P. aeruginosa WIBG2.2	0.44 (0.05)	0.36 (0.09)	0.41 (0.01)	0.77 (0.24)	1.18 (0.58)	0.83 (0.16)	1.06 (0.61)	0.79 (0.28)	0.87 (0.18)
S. aureus WIBG1.2	0.33 (0.08)	0.52 (0.04)**	0.55 (0.03) **	0.48 (0.05)	0.40 (0.02)**	0.40 (0.03) *	1.46 (0.19)	1.75 (0.11)**	1.73 (0.14) *
S. aureus WIBG1.6	0.49 (0.02)	0.60 (0.02) **	0.63 (0.06) **	0.43 (0.02)	0.37 (0.02) **	0.37 (0.03) *	1.60 (0.07)	1.89 (0.12) **	1.89 (0.17) *
MRSA	0.34 (0.03)	0.32 (0.07)	0.42 (0.05)	0.57 (0.15)	0.71 (0.09)	0.51 (0.26)	1.29 (0.31)	0.99 (0.13)	1.30 (0.29)
S. epidermidis	0.22 (0.02)	0.21 (0.01)	0.23 (0.03)	0.53 (0.04)	0.54 (0.06)	0.42 (0.04) **	1.31 (0.10)	1.30 (0.15)	1.66 (0.15) **
S. pyogenes	0.35 (0.02)	0.37 (0.01)	0.44 (0.14)	0.59 (0.13)	0.54 (0.11)	0.48 (0.14)	1.40 (0.42)	1.20 (0.01)	1.61 (1.06)

Significance denoted as * ($P < 0.05$) or ** ($P < 0.01$) following pairwise comparison of P10 or X10 to baseline (P0) growth metric data. Standard deviations are given in the parentheses if the data varied between replicates

4.3.2 Impact of Manuka honey wound gel passaging on bacterial biofilm formation

A crystal violet assay was used to determine biofilm formation for all bacteria before and after repeated wound gel exposure and following ten passages in a honey-free medium (Figure 4.2). Overall, 3/8 strains exhibited significant reductions in biofilm formation following passaging (*S. aureus* WIBG 1.2, MRSA and *S. pyogenes*). In contrast, 4/8 strains, including *S. epidermidis*, *P. aeruginosa* (WIBG 1.3, 2.2) and *E. coli,* showed a significant increase in biofilm formation according to the crystal violet assay. Reversion to baseline data was observed regarding biofilms formed by X10 *S. epidermidis* and X10 *P. aeruginosa* WIBG 1.3.
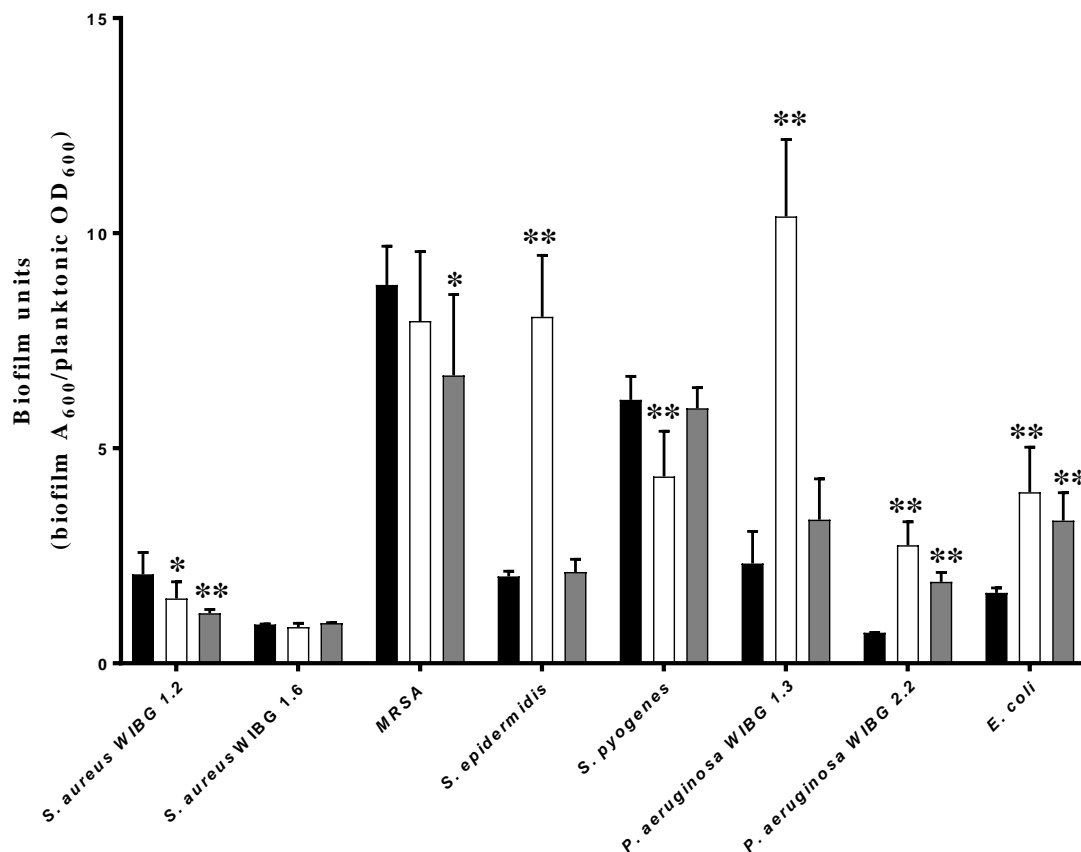
Figure 4. 2 Biofilm formation in parent (P0, black) and passaged (P10, white, X10, dark grey) bacteria following adjustment for planktonic mass. Significant differences in biofilm formation following pairwise comparison with parent are denoted as * and ** (P ≤ 0.05 and 0.01, respectively). Error bars denote standard deviation.

4.3.3 Relative pathogenicity of passaged bacteria

A *Galleria mellonella* waxworm model was used to determine relative pathogenicity in all tested bacteria (Figure 4.3). P10 passaged strains exhibited increased virulence (P ≤ 0.05, log-rank test) in 3/8 bacteria (*S aureus* WIBG 1.2, *S. epidermidis* and *P. aeruginosa* WIBG 1.3; Figures 4.3 A, D, G) when compared to parent strains (P0). These changes in pathogenicity were transient with partial or complete reversion in the absence of continued antimicrobial exposure (X10). A small but significant (P= 0.049) increase in larval killing was also observed in *E. coli* (X10) when compared to baseline data (Figure 4.3 F). In contrast, a significant attenuation in pathogenicity was observed in *S. aureus* WIBG 1.6 following ten passages (Figure 4.3 B).
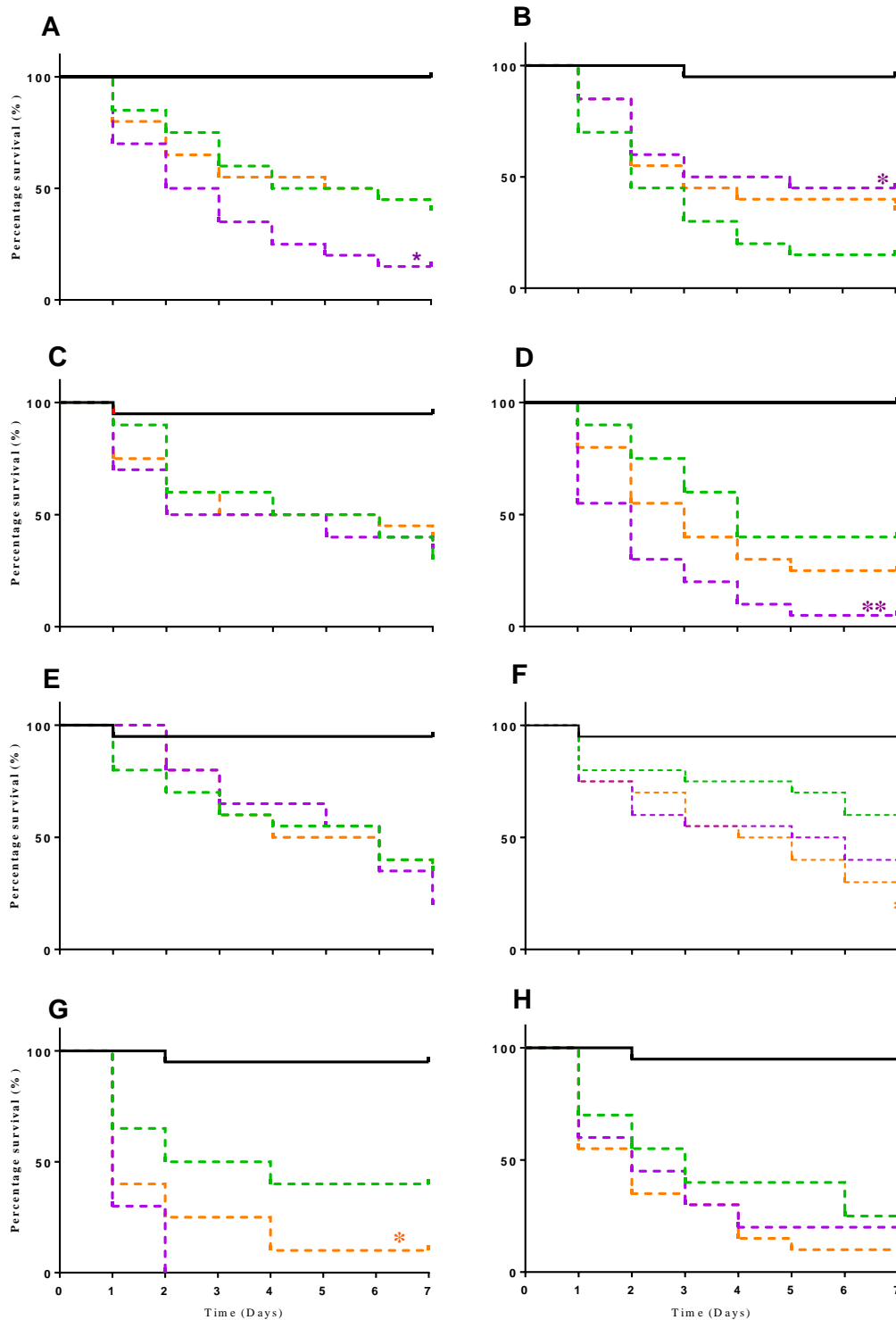
Figure 4. 3 Kaplan Meir curve illustrating percentage survival following injection of Galleria mellonella (wax moth) with sterile PBS (solid black line), parent (P0, green dotted line), passaged (P10, purple dotted line), and X10 (orange dotted line) bacteria. Each curve represents a different test bacterium as follows: *S. aureus* WIBG 1.2 (A), *S. aureus* WIBG 1.6 (B), *MRSA* (C), *S. epidermidis* (D), *S. pyogenes* (E), *E. coli* (F*), P. aeruginosa* WIBG 1.3 (G), *P. aeruginosa* WIBG 2.2 (H). Significant differences in virulence following pairwise comparison with parent strain denoted as *and** (P ≤0.05 and 0.01, respectively).

4.3.4 Bacterial phenotypic characteristics after Manuka honey wound gel exposure

4.3.4.1 Loss of pigmentation and colony variation in *S. aureus* WIBG 1.6

After continued passaging against Manuka honey wound gel, *S. aureus* WIBG 1.6 exhibited a loss of pigmentation (Figure 4.4). In addition, colonies exhibited a significant increase in mean colony size when comparing P10 (1089.6 µm ± 312.5, P< 0.0001) with both parent (400.18 µm ± 88.5) and X10 (662.85 µm ± 142.2) (Figure 4.5).
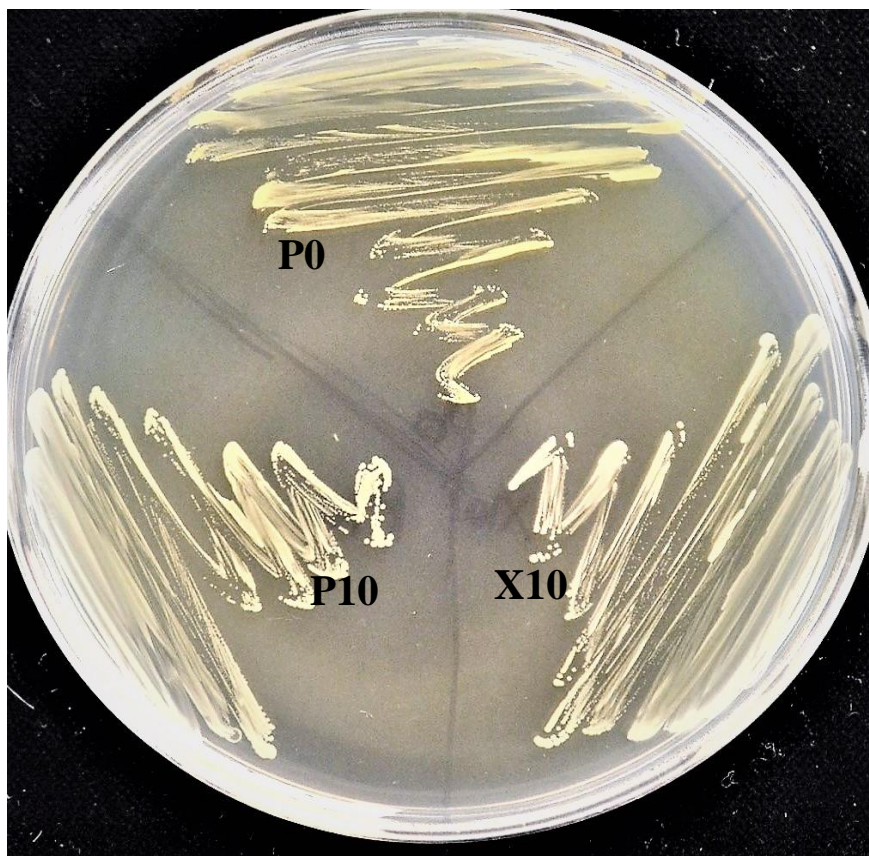
Figure 4. 4 Loss of colony pigmentation of *S. aureus* WIBG 1.6 after exposure to Manuka honey wound gel (P10 and X10).
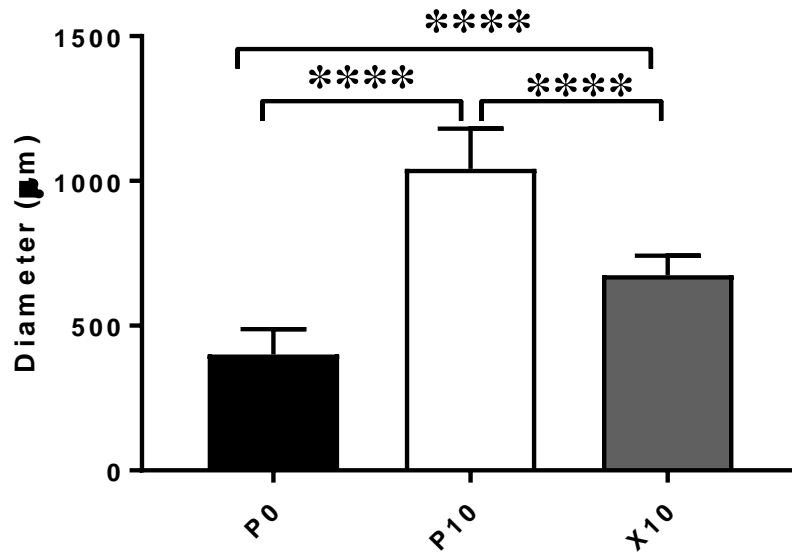
Figure 4. 5 Average colony diameter of *S. aureus* WIBG 1.6 parent strain (P0) and the honey adapted strains (P10 and X10) measured using ImageJ analysis software. Error bars show standard deviation (3 biological, each with 3 technical replicates).

4.3.4.2 DNase activity

S. aureus WIBG 1.6 exhibited a decrease in virulence, thus, the capability of *S. aureus* WIBG 1.6 to hydrolyse DNA was examined before (P0) and after Manuka honey wound gel exposure (P10 and X10) using a DNase agar (Oxoid, UK). All strains of *S. aureus* exhibited DNase activity demonstrated by a clear zone of clearance around bacterial colony-forming units following the addition of hydrochloric acid to the media surface (Figure 4.6). Interestingly, *S. aureus* WIBG 1.6 (P10) exhibited a smaller zone of clearing (13 mm ± 1.89) compared to both the parent strain (22.8 mm ± 2.04, P<0.0001) and X10 strain (27.5 mm ± 2.07, P= 0.0028).

Figure 4. 6 Representative DNase activity of *S. aureus* WIBG 1.6 parent strain (P0), Manuka honey wound gel exposed (P10), and following a further ten passages on wound gel honey-free media (X10).

4.3.4.3 *In vitro* coagulase activity of passaged staphylococci

The levels of coagulase produced by planktonic staphylococci were investigated using a tube coagulase test. After repeated passage with Manuka honey wound gel, *S. aureus* WIBG 1.6 showed a delay in coagulation activity in strains P10 and X10, with both showing a positive result after 3 h compared to the parent, the latter exhibiting a positive result after 30 min incubation (Table 4.2). Both WIBG 1.2 and MRSA showed no observable change in coagulation over time. *S. epidermidis* (negative control) exhibited no observable coagulase activity.

Table 4. 2 Coagulase activity of parent and passaged staphylococci.

Bacteria	Time (h)	Activity for passage		
		P0	P10	X10
S. aureus **WIBG 1.2**	0.5	++	++	++
	1	+++	+++	+++
	2	++++	++++	++++
	3	++++	++++	++++
S. aureus **WIBG 1.6**	0.5	++++	-	-
	1	++++	-	+
	2	++++	++	+++
	3	++++	++++	++++
MRSA	0.5	++	++	++
	1	+++	+++	+++
	2	++++	++++	++++
	3	++++	++++	++++
S. epidermidis	0.5	-	-	-
	1	-	-	-
	2	-	-	-
	3	-	-	-

Tubes were observed for signs of coagulation over 3 h and scored on a five-point scale according to the manufacturer's guidelines as follows: -, no coagulation detected; +, small separate clots; ++, small, combined clots; +++, extensively coagulated clots; ++++, complete coagulation

4.3.4.4 Changes in haemolytic potential following passage

The ability of planktonic isolates to lyse erythrocytes was investigated in all passaged isolates that demonstrated: (i) a significant change in pathogenicity assay according to log-rank testing; (ii) observable haemolytic activity when incubated on blood supplemented agar.

4.3.4.4.1 *S. aureus* **WIBG 1.6**

The capability of planktonic P0, P10 and X10 isolates of *S. aureus* WIBG 1.6 to lyse erythrocytes were studied. Higher readings of absorbance are indicative of red blood cells lysis and release of haemoglobin into the supernatant. As shown in Figure 4.7, attenuated haemolysis was noted in *S. aureus* WIBG 1.6 following wound gel passaging (P10) equivalent to 50% that

of the progenitor strain, which was a statistically significant reduction (P= 0.001). After removal of honey, partial reversion in haemolytic activity was noted in X10 (73% of the P0 value, P<0.01).
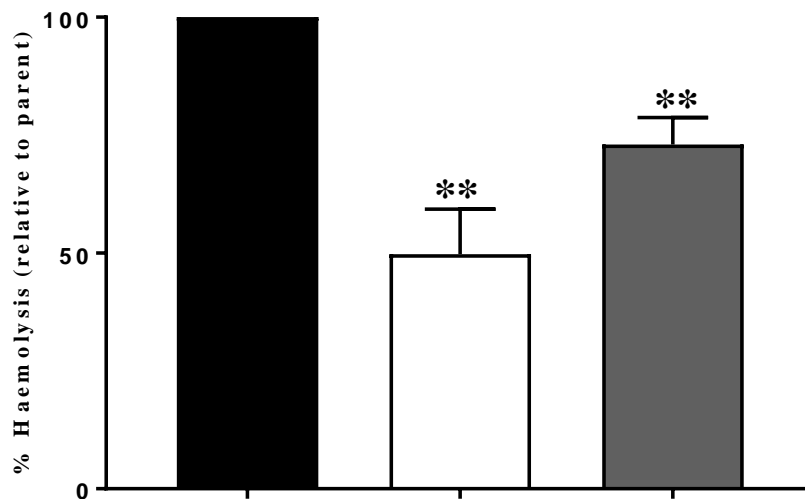
Figure 4. 7 Haemolytic potential of *S. aureus* WIBG 1.6 parent strain (P0, black) and Manuka honey wound gel passaged (P10, white; X10, grey). Data expressed as mean percentages of the P0 value. Error bars show standard deviation (n=4).

4.3.4.4.2 *S. aureus* **WIBG 1.2**

S. aureus WIBG 1.2 P0, P10 and X10 strains were tested for haemolytic activity. After exposure to Manuka honey wound gel, *S. aureus* (P10) showed a significant increase in haemolytic activity (138.3%, P<0.05) relative to the progenitor. However, such an effect was transient, as the X10 strain exhibited 95% of haemolysis relative to the parent (Figure 4.8).
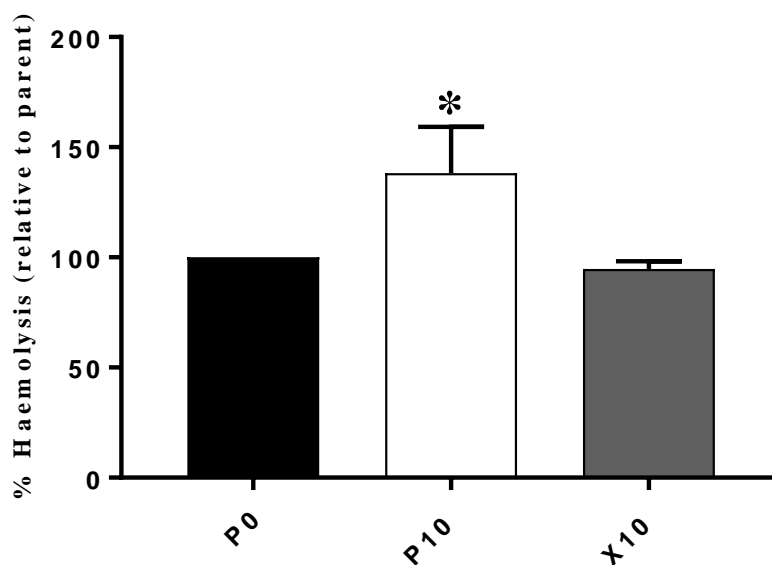
Figure 4. 8 Haemolytic activity of *S. aureus* WIBG 1.2 before and after passages with Manuka honey wound gel. Data presented as a mean percentage of the P0 value. Error bar represents standard deviations (n=4).

4.3.4.4.3 *S. pyogenes*

The haemolytic ability of *S. pyogenes* at P0, P10 and X10 was investigated in this study (Figure 4.9). A sustained increase in haemolytic activity was observed after ten passages with Manuka honey wound gel (138% of the P0 value, P< 0.05). After removal of honey, this observation was sustained with passage X10 demonstrating haemolytic capability equivalent to 135% of the respective parent strain (P<0.05).
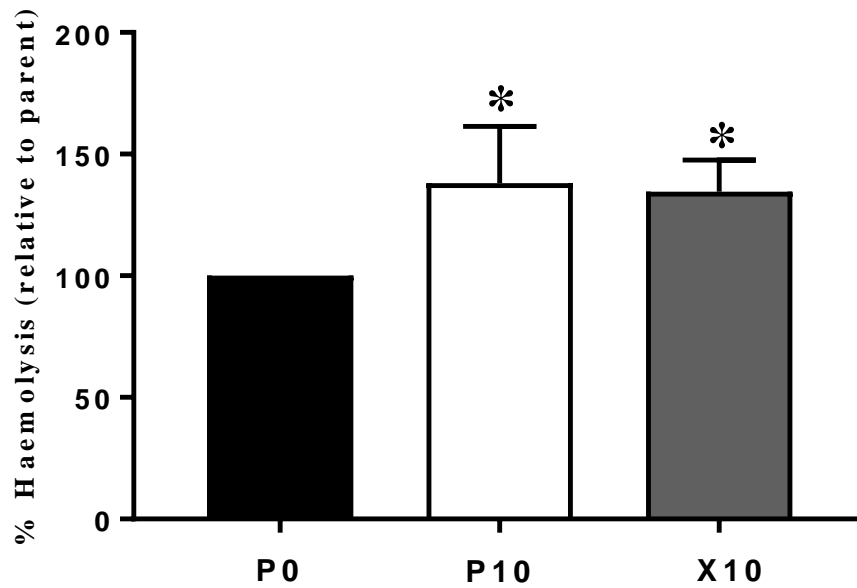
Figure 4. 9 Haemolytic activity of *S. pyogenes* parent strain P0 (black bar); honey-exposed strain (P10, white bar) and a further ten passages on honey-free medium (X10, grey bar). Data are expressed as the mean percentage of the mean P0 value. Error bars show standard deviation (n=4).

4.3.4.4.4 *S. epidermidis*

In this chapter, an increase in haemolytic potential was observed for *S. epidermidis* after ten passages with honey (135% of the P0 value, $P<0.05$). Such observations, however, were transient and marked by a small but significant reduction in haemolytic potential in strain X10 (78% of the P0 value, $P<0.05$) (Figure 4.10).
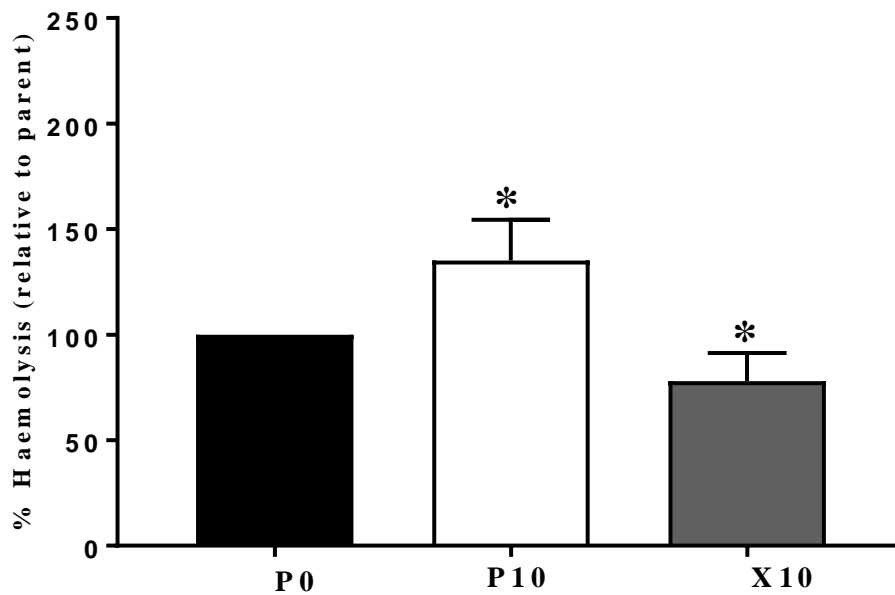
Figure 4. 10 Haemolytic activity of *S. epidermidis* P10 and X10 strains expressed as a mean percentages of the P0 value. Error bars show standard deviation (n=4).

4.3.4.4.5 *P. aeruginosa* WIBG 1.3

The ability of *P. aeruginosa* isolates to lyse erythrocytes was investigated. In comparison to the parent strain, *P. aeruginosa* WIBG 1.3 (P10) showed a significant and sustained increase in haemolytic activity following passaging in the presence of Manuka honey wound gel (130% of the P0 value, P= 0.001) (Figure 4.11). After removal of honey, the observed increase in haemolytic activity was maintained in passage X10 (191% of the parent strain, P=0.004).
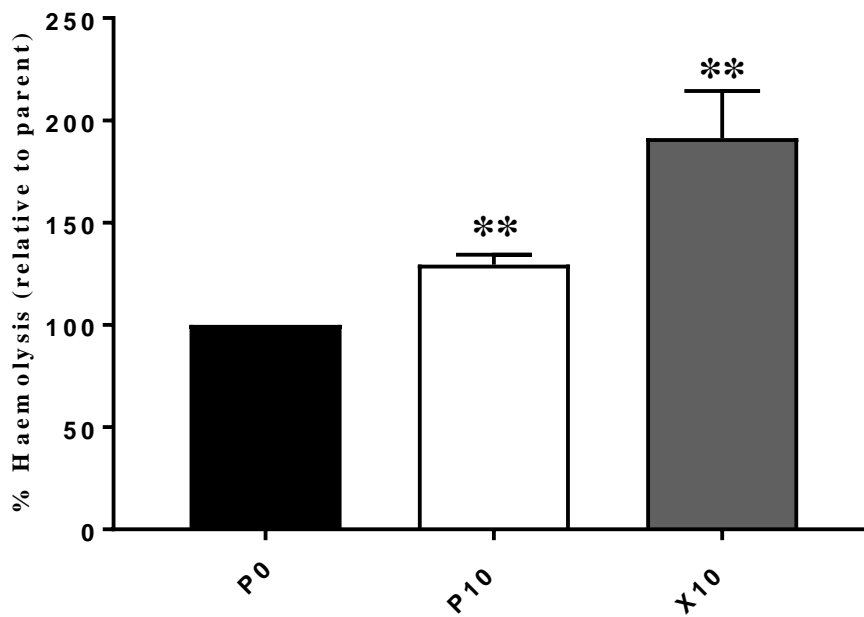
Figure 4. 11 The haemolytic potential of *P. aeruginosa* WIBG 1.3 parent strain (P0, black bar), after ten passages with Manuka honey wound gel (P10, white bar) and further ten passages without honey (X10, grey). Data are relative to the haemolytic activity of the parent strain. Significant changes in haemolytic following pairwise comparison with baseline data are denoted as ** (P< 0.01). Error bars show standard deviation.

4.3.4.5 Pyocyanin production in *P. aeruginosa*

P. aeruginosa WIBG 1.3 showed a significant increase in the production of pyocyanin after repeated exposure to Manuka honey wound gel (P0 vs. P10, 557.5% ± 66.3; P= 0.007). After removal of the antimicrobial challenge, a reversion in pyocyanin production was observed (P0 vs. X10, 221.5% ± 106.1; P=0.19). Pyocyanin production was not observed in *P. aeruginosa* WIBG 2.2 and as such no data is reported for this strain (Figure 4.12).
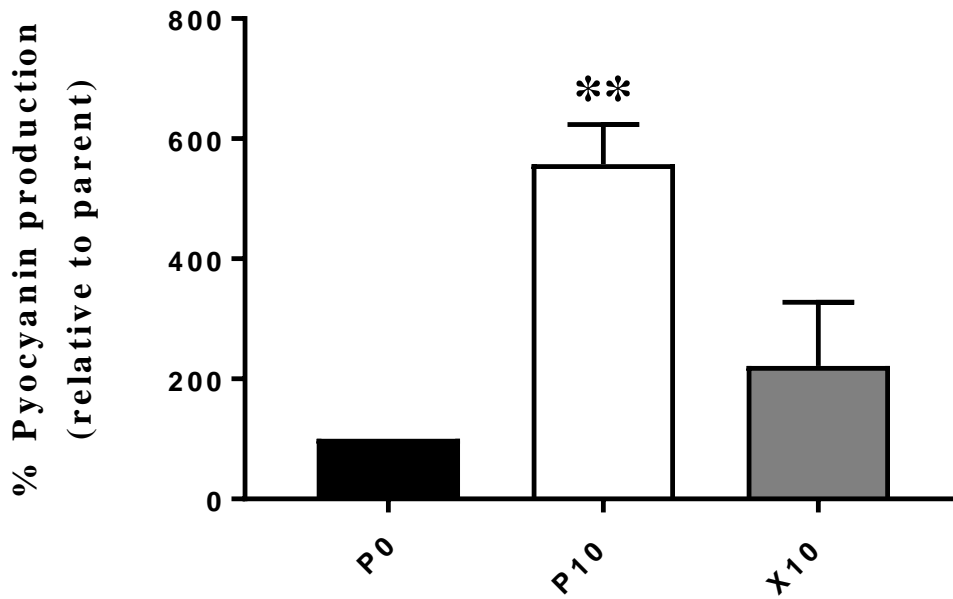
Figure 4. 12 Pyocyanin production by *P. aeruginosa* WIBG 1.3 parent (P0, black) and Manuka honey wound gel passaged (P10, white, X10, dark grey) bacteria. Data are expressed as a percentage respective to the progenitor (P0) strain. Significant data are represented as ** (P< 0.01). Error bars show standard deviation.

4.3.4.6 Changes in motility after Manuka honey exposure in *P. aeruginosa* WIBG 1.3

. After passaging with Manuka honey wound gel, the P10 strain of *P. aeruginosa* WIBG 1.3 showed a significant increase in swimming (67.83mm±7.22, P=0.0023) and swarming (77.67mm±3.38, P< 0.0001) motility compared to P0 (swimming=51.17mm±6.96; swarming=44.5mm±4.32) following 24h incubation. Such changes were, however, transient with partial reversion to baseline levels after honey wound gel removal (X10)(swimming=58.33mm±4.63; swarming=61.67mm±8.95). No significant increase in twitching motility at P10 (15.17mm±3.25) when compared to P0 (13.3mm±2.50) and X10 (14.3mm±2.58) strains ((P> 0.05) (Figure 4.13).
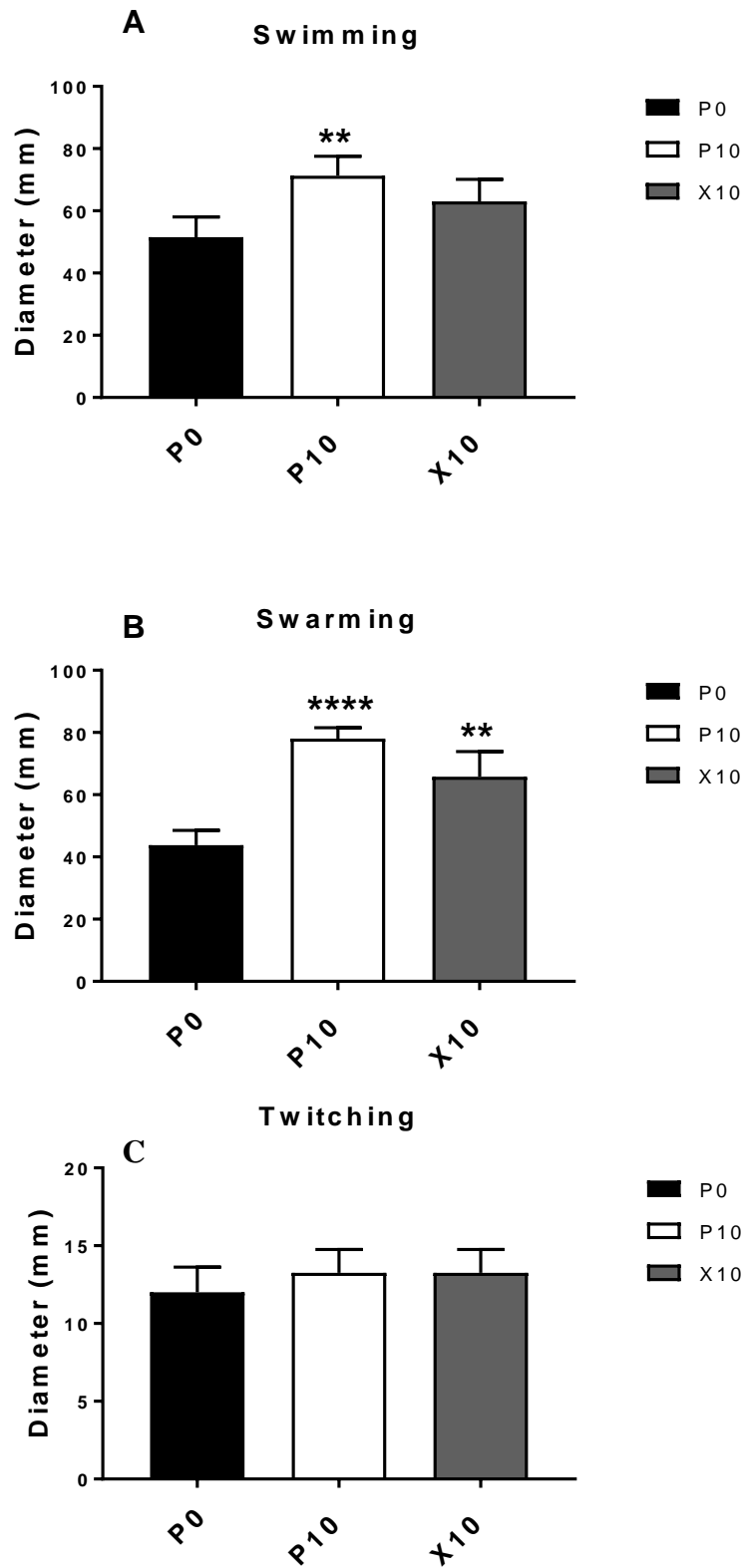
Figure 4. 13 Swimming (A), swarming (B), and twitching motility (C) of *P. aeruginosa* WIBG 1.3 parent strain (P0, black) and Manuka honey wound gel passaged (P10, white, X10, dark grey). The shown values reflect the mean diameter of the corresponding motility zones and the standard deviation of two representative experiments with triplicate plates per experiment is expressed by error bars.

4.4 Discussion

We have previously demonstrated that passaging in the presence of a Manuka honey-based wound gel led to variable limited changes in antimicrobial sensitivity profiles in a panel of chronic wound bacteria. However, the effect of honey exposure on microbial growth and virulence potential, as has been observed in the laboratory through exposure to various biocides, (Latimer et al., 2012, Bayston et al., 2007) is less frequently discussed in the literature. Such adaptations could have substantial effects on wound treatment in a way that is independent of changes in antimicrobial sensitivity.

The use of the *Galleria mellonella* waxworm model in this study enabled an assessment of the virulence potential in a panel of bacterial pathogens, and suggested variable effects on pathogenesis, particularly between members of the staphylococci. In general, altered virulence in this genus occurred in conjunction with changes in growth metrics, haemolytic activity, coagulation and biofilm formation and as such agrees with studies investigating passaging in the presence of other antimicrobials (Latimer et al., 2012, Bazaid et al., 2018). Honey exposed *S. aureus* WIBG 1.6 demonstrated a loss of pigmentation in Mueller Hinton grown colony-forming units after honey exposure, an observation accompanied by a temporary decrease in haemolysin and DNase activity. Previously, the formation of non-pigmented colonies in *S. aureus* has been associated with decreased pathogenicity in a mouse abscess model, which showed that non-pigmented bacteria are more prone to clearance by the immune system when compared to pigmented bacteria (Liu et al., 2005). Moreover, in previous studies exposing *S. aureus* to triclosan, a reduction in the release of virulence factors such as haemolysin and DNase (Bayston et al., 2007, Latimer et al., 2012), has been reported. Haemolytic toxins may be related to delayed wound healing by the disturbance of cell proliferation and migration (Demidova-Rice et al., 2012, Marano et al., 2015), thus, attenuated haemolysin release following honey exposure may have a positive impact on the wound healing process. Similarly,

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the observed reduction in DNase activity could partially account for the reduced virulence of the *S. aureus* WIBG 1.6 as this virulence factor has been linked to biofilm growth (Mulcahy et al., 2010), maturation (Mann et al., 2009) and resistance to the host immune system (Berends et al., 2010).

In contrast to *S. aureus* WIBG 1.6, which demonstrated reduced virulence potential in the laboratory, *S. epidermidis* exhibited an enhanced killing effect in the wax worm model that was concomitant to increased haemolytic activity and biofilm formation potential. After exposure to antimicrobial agents, multiple factors may enhance bacterial virulence, such as increased bacterial growth rate, changes in lipopolysaccharides of the cell wall and the expression of major bacterial virulence factors such as adhesins or toxins (Kastbjerg et al., 2010, Beceiro et al., 2013). Whereas historically only considered a harmless commensal microorganism on human skin, *S. epidermidis* is now seen as an important opportunistic pathogen. It is now the most common source of nosocomial infections, similar to its more virulent relative *Staphylococcus aureus*, at a rate almost as high as that (Gomes et al., 2014, System, 2004). The normal skin microbiota helps the host by competing with pathogens for nutrients, niches, and receptors. Under certain circumstances however, commensal bacteria can overwhelm the host defence and opportunistic infections may occur (Bartold and Van Dyke, 2013). Alteration in the normal skin microbiota, changes in the host immunity and break in the skin integrity can lead to microbial imbalance (dysbiosis) and cause disease, for instance, *S. epidermidis* can cause infections related to medical devices such as endocarditis and surgical wound infections (Kong and Segre, 2012).

Interestingly, the enhanced virulence observed in *P. aeruginosa* WIBG 1.3 (P10) is in contrast to previous reports. For example, *P. aeruginosa* wild-type PA14 has been shown to exhibit reduced pyocyanin production following exposure to both raw and heat-treated Manuka honey, likely via interaction with the MvfR quorum sensing network (Wang et al., 2012). In

the present study, honey adaptation was associated with significant increases in pyocyanin production following chloroform extraction. The potential significance of pyocyanin in virulence and pathogenicity of pseudomonal infections and its potentially toxic effects have been shown in various studies to date (Lau et al., 2004a, Lau et al., 2004b, Smith et al., 2003). Pyocyanin has been shown to have multiple adverse effects including pro-inflammatory and free radical effects resulting in cellular damage and death (Lau et al., 2004b, Britigan et al., 1992, Denning et al., 1998, McDermott et al., 2013). In the present study, *P. aeruginosa* WIBG 1.3 additionally demonstrated significant increases in haemolysin activity, growth rate, motility and biofilm formation at P10, the latter observation in agreement with a previous study (Camplin and Maddocks, 2014). Such observations could explain the increase in MBEC concentration of gentamicin after passaging with Manuka honey, although warrants further investigation (Section 3.3.1.2).

P. aeruginosa has three movement styles depending on medium viscosity: (i) swimming in aqueous settings; (ii) twitching on solid surfaces, and (iii) swarming on viscous semi-solid media (0.4-0.7% w/v agar). Under swarming conditions, significant changes in gene expression patterns have been previously observed, including the over-expression of a large number of virulence-related genes, such as that encoding the type-three secretion system, those encoding extracellular proteases, and those associated with the transport of iron (Overhage et al., 2008). In contrast with their planktonic (swimming) counterparts, swarming cells have also demonstrated adaptive antibiotic resistance to polymyxin B, gentamicin and ciprofloxacin by upregulating the multidrug-efflux pump, MexGHI-OpmD (Overhage et al., 2008). In addition, several studies have shown that swarming motility could play a key role in early biofilm formation (Overhage et al., 2007, Shrout et al., 2006). Thus, the increase in swarming motility observed in this chapter could explain the enhanced virulence in *P. aeruginosa* WIBG 1.3. However, it must be noted that enhanced virulence may differ between strains as no significant

changes in killing were observed in WIBG 2.2, supporting the view that honey is a complex compound comprising active elements capable of affecting multiple cellular target sites (Jenkins and Cooper, 2012). Whilst previous reports suggest the antimicrobial activity of honey to vary significantly between species, such observations may also be true for phenotypic adaptation between strains.

The data in this chapter indicate that bacterial exposure to sub-lethal concentrations of Manuka honey wound gel resulted in phenotypic changes including variable changes in pathogenicity, planktonic growth rate, biofilm formation and haemolysin activity. Biofilm formation was variably affected in passaged bacteria, with notable increases in the pseudomonads and *S. epidermidis*. Whilst this could have implications for the treatment length, given the propensity for biofilms to form in wounds, significant changes in biofilm sensitivity were generally limited, as noted in Section 3.3.1.2.

Chapter 5

Adaptation of biofilm derived bacteria following exposure to a Manuka honey wound gel

Abstract

Background. Bacteria within chronic wounds are frequently organized into well-defined microbial communities, termed biofilms, which offer the residing microorganisms resistance to antimicrobial treatment and host immune factors. We have previously established that in planktonic form, specific strains of *P. aeruginosa* and *S. epidermidis* exhibited highly significant increases in biofilm formation and virulence following passaging with a Manuka honey wound gel. Here, we investigate whether such changes are observed in bacteria exposed in biofilm growth modes using a combination of phenotypic and genotypic profiling. **Methods.** Using an MBEC assay^TM, chronic wound isolates of *P. aeruginosa* and *S. epidermidis* were grown in sessile form and passaged through sub-MBEC concentrations of Manuka honey wound gel. To evaluate changes in colony morphology and antimicrobial susceptibility, sample pegs were removed daily and plated onto Mueller-Hinton agar. All isolates were subjected to whole genome sequencing in order to detect mutations in biofilm-derived variants. Additionally, pathogenicity, biofilm formation ability and exotoxin production were assessed in all variants of *P. aeruginosa* and *S. epidermidis*. **Results.** Following honey exposure, colonies with reduced diameter were cultured from passaged biofilms of both *P. aeruginosa* and *S. epidermidis* and exhibited reduced sensitivity to gentamicin and vancomycin, respectively, when compared to controls. Whole genome sequencing of a *P. aeruginosa* biofilm-derived colony variant identified a point mutation in three different coding genes including *cheB_1*, *hudA* and *lasR* when compared to passage control variants, while point mutations in *cdaR*, *sdrG*, *scrK* and *lipA* genes were noted in *S. epidermidis* passaged isolates relative to controls. Virulence was significantly increased ($P < 0.05$) in biofilm derived bacteria following wound gel passaging when investigated using a non-mammalian virulence model. Enhanced virulence in passaged *P. aeruginosa* was observed in conjunction with increased biofilm formation and overproduction of extracellular protease, elastase and pyocyanin but demonstrated reduced swarming and twitching motility. **Conclusion.** Biofilm passaging with a Manuka honey wound gel led to genotypic and phenotypic changes that should be considered when treating chronic wound infections.

5.1 Introduction

Chronic ulcers are those that do not heal in a timely manner (3 weeks to 3 months) and have become a major problem for healthcare systems around the world (Howell-Jones et al., 2005a). They affect about 3% of people over the age of 60 (Davies et al., 2007) and are usually caused by neuropathy (diabetic and pressure ulcers), vasculopathy (venous and arterial ulcers), or trauma (Lipsky and Hoey, 2009). Delayed wound healing is often caused by bacterial infection and hindering treatment of such infections increases risk of morbidity and mortality (Landis et al., 2007). Initially, it was believed that wound infection resulted from the colonization and invasion of free-floating planktonic bacteria (Edwards and Harding, 2004). However, research in the last few decades has established the role of the biofilm and its influence on chronic wound healing (Wolcott et al., 2009). Molecular sampling techniques have proven that chronic wounds contain a more distinct population of microorganisms than previously thought (Dowd et al., 2008).

Biofilms are an aggregation of microbial cells that are embedded within a self-produced layer of extracellular polymeric substance (Hurlow et al., 2015, Jamal et al., 2018). Biofilm cells have varying physiological and metabolic properties, distinct from those of planktonic cells, and in particular are widely reported to offer recalcitrance to antimicrobial exposure. Although the biofilm matrix is traditionally considered a key contributor, the mechanisms underpinning biofilm recalcitrance are likely multifactorial and may involve the development of physiological gradients (Walters et al., 2003, Høiby et al., 2010), reaction diffusion limitation (Stewart et al., 2016, Roberts and Stewart, 2004, Stewart, 2002) and/or the presence of biofilm-specific phenotypes (Percival et al., 2011, Humphreys and McBain, 2014). Antimicrobial activity is well-known to be dependent on the metabolic state of the bacteria, the physiochemical properties of the microenvironment (such as oxygen and nutrients), and the presence of specific phenotypes with low metabolic activities in biofilms, including small

colony variants (SCVs) and persister cells (Conlon et al., 2015, Garcia et al., 2013). To this end, Manuka honey has shown anti-biofilm activity. Previous studies have documented that Manuka honey at a concentration of 16% w/v is capable of eradicating bacterial biofilms of both *P. aeruginosa* and *S. aureus in vitro* (Lu et al., 2014, Lu et al., 2019). The underlying mechanism of preventing biofilm formation in *P. aeruginosa* has been investigated and reported to be due to fructose, which is able to bind to Pseudomonas Lectin II (LecB) and consequently prevents biofilm establishment (Lerrer et al., 2007). Whilst there is limited evidence from controlled trials regarding advanced antimicrobial dressings in wound management, they are frequently applied in the NHS, accounting for an NHS expenditure in excess of £28 million (1997-2016), of which, honey containing dressings account for approximately 23% of such costs (Gray et al., 2018, Hussey et al., 2019).

Long-term bacterial exposure to sub-inhibitory concentrations of antimicrobial agents may induce antimicrobial insusceptibility by selecting intrinsically resistant mutants or induced phenotypic adaptations (Forbes et al., 2014, Henly et al., 2019). This phenomenon could theoretically occur more rapidly in the biofilm mode of growth, where bacteria are exposed to sub-lethal concentrations of antimicrobial through the presence of physiological gradients. Previous studies have suggested that neither *S. aureus* nor *P. aeruginosa* planktonic cultures develop resistance to honey after continued sub-culture in the presence of sub-inhibitory concentrations of Manuka honey (Blair et al., 2009, Cooper et al., 2010b). In contrast, the exposure of pseudomonad biofilms to Manuka honey wound gels have yielded slow growing isolates with marginal decreases in sensitivity to honey, imipenem and rifampicin (Camplin and Maddocks, 2014).

We have previously demonstrated that in a planktonic form, specific strains of *P. aeruginosa* and *S. epidermidis* exhibit decreased sensitivity to antibiotics with increased biofilm formation and virulence potential following honey exposure. Previous research has

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evaluated the impact of prolonged honey exposure on the bacterial susceptibility of planktonic cells (Blair et al., 2009, Cooper et al., 2010b), but there are limited investigations regarding potential adaptations that may occur in bacteria exposed in biofilm growth modes. Here, we investigate such effects using a hight throughput, reproducible biofilm system in conjunction with phenotypic characterisation and genome sequencing.

5.2 Materials and methods:

5.2.1 Bacterial strains, Growth conditions and Chemical reagents

Both *P. aeruginosa* WIBG 1.3 and *S. epidermidis* ATCC 14990 isolates were used as a part of a previous study investigating adaptation in planktonic isolates (Mokhtar et al., 2020). Bacteria were maintained on Mueller-Hinton agar and Mueller-Hinton broth (Oxoid, Basingstoke, UK). Medihoney antibacterial wound gel (20g tube) was obtained from Derma Sciences (Berkshire, UK). Antimicrobial agents including gentamicin, meropenem and vancomycin were obtained from Sigma-Aldrich (Dorset, UK). Ciprofloxacin was purchased from Alfa Aesar (Lancashire, UK). Stock solutions of Medihoney Medical grade wound gel (Derma Sciences, Berkshire, UK) and all antibiotic stocks were freshly prepared using distilled water and sterilised by filtration (0.22µM, Millipore, Watford, UK).

5.2.2 Biofilm Culture

An overnight culture was prepared for each bacterium under aerobic conditions (12h, 37ºC), adjusted to an OD_{600} of 0.8 and further diluted 1:100 into Mueller Hinton Broth. A 100 µl aliquot of diluted inoculum was placed into each well of an MBEC assay™ device (Fisher scientific, UK) and incubated at 37°C for 48 h to support biofilm formation. After incubation,

pegged lids were washed twice in 200 µl of sterile phosphate-buffered saline and transferred to antimicrobial exposure plates comprising increasing Manuka honey concentrations across the ordinate (5-25%w/v, 5% interval) for *S. epidermidis* and (5-35%w/v, 5% interval) for *P. aeruginosa*. MBECs were performed at the beginning of the experiment to determine the concentration and for every passage to know at which honey concentration the pegs for colony morphology assessment were taken from the well (5% below the MBEC level). Antimicrobial plates were incubated aerobically at 37°C for a further 24h and was defined as a single biofilm passage (BP). This procedure was repeated until reaching the fifth biofilm passage (BP5). Control exposures comprising microbiologically sterile Mueller Hinton broth only were performed in parallel to account for biofilm heterogeneity under laboratory conditions (BC5).

5.2.3 Colony morphology assessment

In order to evaluate the variation in colony morphology, three pegs were detached using a sterile flamed scalpel. Passage experiments were conducted as biological duplicates. Each peg was transferred into 10 ml PBS, vortexed and serially diluted (1 in 10) to a final dilution of 10^{-6}. Aliquots (100 µL) of each dilution were cultured onto Mueller Hinton agar and incubated at 37°C for 24 h. Colony morphology was examined daily for changes in size, colour, and regularity. Images were taken using a Nikon digital camera (D3200). Colony diameters were measured through image analysis in ImageJ (National Institutes of Health) and were expressed as means (mm) of biologically two experiments, each comprising 6 technical repeats. The biofilm-derived variants were immediately stored at -80°C in the absence of further subculture for genomic study.

5.2.4 Determination of bacterial MBECs

Minimum biofilm eradication concentrations (MBECs) were performed for all isolates and control exposures in order to determine the concentration of antibiotic and Manuka honey to eradicate the biofilm. MBECs were performed as described previously (Ceri et al., 1999). Briefly, overnight bacterial cultures were adjusted to an OD_{600} of 0.8, further diluted 1 in 100 and 100 µL volumes were dispensed into each well of the MBEC assay^{TM} plate. The plate was incubated at 37°C for 48 h to allow biofilm formation. After the incubation period, the transportable pegged lid was detached, rinsed twice with sterile phosphate-buffered saline (PBS) and transferred to an antimicrobial challenge plate containing either doubling dilutions of antibiotic (2000 – 0.03 mg/L) or 5% (w/v) increments of Manuka honey wound gel across the wells. The plate was incubated for 24 h at 37°C, then, the pegged lid was transferred to a recovery plate that contained 200µL of sterile broth, sonicated (*c.* 50 kHz, 5 min) using a sonicor (model SC-52TH) and incubated for 24 h at 37 ˚C. The minimum biofilm eradication concentrations (MBECs) were defined as the lowest concentration of antimicrobial necessary to kill a biofilm. The turbidity in wells indicated bacterial growth in comparison to the clear negative control wells. The data represents the mean and standard deviation of two separate experiments with each comprising three technical replicates.

5.2.5 Whole genome sequencing and data analysis

DNA extraction and whole genome sequencing (WGS) of the parent strains (BP0) and biofilm-derived variants (BP5 and BC5) were performed at the MicrobesNG Facility, University of Birmingham, UK, using the Illumina HiSeq platform. Sequences were analysed through several pipelines; starting by using Kraken to identify the closest reference genome, which confirmed that all sequences are *S. epidermidis* and *P. aeruginosa*. The data was then

de novo assembled using SPAdes (http://bioinf.spbau.ru/spades) followed by variant calling against the closest reference genome (*P. aeruginosa* DK1-NH57388A; *S. epidermidis* IS-K). Nonsynonymous mutations were detected by mapping the biofilm-derived variant sequences against the untreated parent strain (BP0) sequence.

5.2.6 *Galleria mellonella* pathogenesis assay

Bacterial virulence was determined as described previously (Mokhtar et al., 2020). Briefly, overnight cultures were grown in Mueller Hinton broth and washed twice in sterile Phosphate Buffer Solution then adjusted to an OD_{600} corresponding to a standard inoculum of bacteria (OD_{600}=0.1 followed by 1:100000, 5.44x10^6 CFU/ml for *S. epidermidis,* OD_{600} =0.1 followed by 10^{10} dilutions, 46 CFU/ml for *P. aeruginosa*). A Hamilton syringe was used to inject 5 µL of the adjusted bacterial suspension into the hemocele of each *G. mellonella* through the rear left proleg. Untreated larvae and larvae injected with sterile PBS were used as control groups. All groups were incubated in petri dish at 37 ˚C, and death was recorded daily. The experiments were terminated when 2 of the control groups had died or after 7 days of incubation post bacterial exposure. Two separate biological replicates were performed for each strain. The data were presented as a Kaplan-Meier survival curves and pairwise comparisons of datasets conducted using the log-rank test using Graph Pad Prism 7 (GraphPad Software, California, USA).

5.2.7 Dynamic growth rate

Bacteria isolated from unexposed (BP0), passage exposed (BP5) and passage control (BC5) were grown in Mueller Hinton broth overnight (37degC, 12h). The OD_{600nm} of cultures was adjusted to 0.8, then diluted 1 in 100 before being deposited in to 96 wells plates (150 µl). The

culture plate was placed into a microplate reader (PowerWave[TM] XS, BioTek, Swindon, UK) and the optical density was read every hour for up to 30 h for *P. aeruginosa* and *S. epidermidis* using Gen5[TM] 1.08 software (BioTek, Bedfordshire, UK). Using the R software package Growthcurver (Sprouffske and Wagner, 2016), growth curve data from eight absorbance readings were adapted to a basic form of the logistic equation to determine the intrinsic growth rates (*r*), carrying capacity (*K*) and maximum generation time (t_gen). The generated data were statistically analysed, and comparisons were performed between parent, passaged mutants, and controls (BP0 vs BP5; BC5 vs BP5) at $P < 0.05$ using a Wilcoxon signed-rank test. Statistical comparisons were performed in GraphPad Prism version 7 (GraphPad Software, California, USA).

5.2.8 Crystal violet biofilm assay

The crystal violet assay was adapted from O'Toole (O'Toole, 2011). To determine the growth of biofilms, overnight bacterial cultures were adjusted to an OD_{600} of 0.8 and then further diluted 1 in 100 in sterile Mueller Hinton broth. Aliquots (150 µl) of diluted inoculum were deposited into 96 well microtiter plate and were incubated aerobically at 37 ˚C for 48 hrs. Following incubation, the liquid culture was aspirated, and the wells were washed twice using 200 µl of sterile phosphate-buffered saline (PBS). A solution of 1% (250 µl) crystal violet was added to each well for 1 min, washed twice with 200 µl PBS and left to dry at room temperature. Thereafter, 300 µl of absolute ethanol was added to each well and incubated at room temperature for 10 min in order to solubilise the attached crystal violet. The absorbance (OD_{600}) was determined for each well relative to sterile control using a PowerWave[TM] XS plate reader (BioTek, Swindon, UK). Data were analysed using GraphPad Prism version 7.0 (GraphPad

Software, California, USA) and are representative of two biological and eight technical replicates.

5.2.9 Pyocyanin assay

Pyocyanin activity was measured for all *P. aeruginosa* biofilm derived bacteria (BP5, BC5) and compared to the parent strain. It was performed as described by Essar et al (Essar et al., 1990) with some modifications. Overnight cultures were grown in 10 ml of PB medium (20g Bacto peptone, 1.4 g $MgCL_2$, 10 g K_2SO_4) to maximize pyocyanin production. Overnight suspensions of BP0, BP5 and BC5 variants were centrifuged (13,000 rpm,10 mins) to obtain the supernatant. The pyocyanin was extracted from a chloroform layer following vigorous agitation with re-extraction achieved using 0.2N HCl. Optical density measurements of the extracted solution were determined using a light spectrometer at 520 nm and the data were expressed as microgram of pyocyanin per millilitre of supernatant by multiplying the optical density by 17.072 (Kurachi, 1958, Essar et al., 1990).

5.2.10 Motility test for *P. aeruginosa* WIBG 1.3

All procedures were performed at room temperature unless specified otherwise. The following protocol is scaled to make 1 L batch of agar. The swim plates comprised 15 g of Luria broth and 3 g of nutrient agar while swarm plates comprised 8 g of nutrient broth, 50ml 10% glucose and 5 g of nutrient agar. All plates were inoculated with an overnight culture of *P. aeruginosa* using a sterile pipette tip (37 ˚C; 24 h). Twitching motility was assessed using 25 g of Luria broth with 10 g nutrient agar, stab inoculated to the base of the petri dish with an overnight culture using a sterile toothpick and incubated for 24 h at 37 ˚C. Following incubation, the agar was removed, and the petri dish was stained with crystal violet for 2 min.

The diameters of motility zones were determined by taking the mean of two perpendicular measurements.

5.2.11 Protease Azocasein Assay

Proteolytic activity of *P. aeruginosa* biofilm culture was assessed by the azocasein procedure according to Schmidtchen et al (Schmidtchen et al., 2001). Culture supernatant (30 µl) were added to 50 µl of azocasein substrate (2% azocasein in 10mM Tris HCL, 8mM CaCl$_2$, pH 7.4) and incubated at 37 ˚C for 3h. Thereafter, the reaction was stopped with 240 µl 10% trichloroacetic acid (TCA) and incubated for 15 mins at room temperature to ensure full precipitation of the undigested material. In the blank assay, only azocasein substrate was incubated at 37 ˚C for 3h and TCA was added to stop the reaction. After centrifugation of the reaction mixture at 10,000 rpm (10 mins), the supernatant was transferred to tubes containing 280 µl of 1 M NaOH and the absorbance at 440 nm was measured. Proteolytic activity was defined as the difference between the absorbance of the assay and the assay blank. The average optical density was calculated for two individual experiments each comprising four technical repeats.

5.2.12 Elastase Congo-red Assay

The elastase activity of *P. aeruginosa* was determined as described elsewhere (Calfee et al., 2001). Overnight bacterial cultures were centrifuged at 10,000 rpm for 10 mins to recover the supernatant. Then, the supernatant was passed through a 0.45-µm syringe filter (Sigma-Aldrich, Dorset, UK). Filtered supernatant (1ml) was added to 1 ml of elastase congo-red (ECR) buffer (0.1 M Tris-HCL, 0.1Mm CaCl$_2$, pH 7.2) and 20 mg of ECR (Sigma-Aldrich). Tubes were incubated for 3h at 37 ˚C with shaking (150 rpm). After incubation, 0.12 M

Na$_2$EDTA (0.2 ml) was added to stop the reaction and the insoluble ECR removed by centrifugation (3,500 rpm, 10 min). The absorbance of the supernatant was determined at 495 nm with data representative of 2 biological experiments, each comprising four technical repeats. The optical density of samples incubated in the absence of supernatant was considered as a blank, and this value was subtracted from all samples.

5.2.13 Screening and quantification of Lipase activity in *S. epidermidis* ATCC 14990

A sensitive and specific plate assay for the detection of lipase producing *S. epidermidis* was utilised that was based upon the use of Rhodamine-olive oil-agar medium (Ameri et al., 2015, Rabbani et al., 2013, Alhamdani and Alkabbi, 2016). The pre-poured media contained the following (/L): nutrient broth 8g, NaCl 4g and nutrient agar 20g. The medium was adjusted to pH 7, autoclaved and cooled to about 60˚C before added 31.25ml of olive oil and 500 µl of rhodamine B solution (0.01%w/v distilled water and sterilized by filtration) with vigorous stirring. Under aseptic conditions, the molten agar was poured into petri plates and allowed to solidify before inoculation with bacterial cultures. Lipase producing strains were identified on spread plates after incubation at 37 ˚C for 48h. The formation of orange, fluorescent halos or the presence of orange fluorescent colonies when observed under UV illumination was indicative of lipase producing strains (Akanbi et al., 2010, Duza and Mastan, 2014). To quantify lipase activity, a circular well (4-mm diameter) was made in the centre of the plate and filled with 10 µl volumes of liquid bacterial cultures (overnight culture were diluted 1:100, incubated for 12h at 37 ˚C, shaking at 140 rpm) and incubated at 37 ˚C for 24 hr. The diameter of the zone of intensification was measured and the mean of six technical and two biological replicates was determined.

5.2.14 Secondary screening and quantification of lipolytic bacterial strains

The lipolytic bacteria detected using olive oil plates were additionally screened through the appearance of white zones when cultured using a selective medium that utilised Tween80 as a substrate. This agar comprised the following (/L): peptone 10g , NaCl 5g, CaCl.2H2O 0.1g, nutrient agar 20g and Tween 80, 10 ml (1%v/v) (Duza and Mastan, 2014, Alhamdani and Alkabbi, 2016). Tween80 was sterilized by filtration and added to the medium after autoclave process (at 121 ˚C,15 mins and cooled to 45 ˚C). Overnight precultures of *S. epidermidis* strains grown in Muller Hinton broth at 37 ˚C were diluted 1:100 and incubated for 12 h at 37 ˚C with shaking at 140 rpm. A circular well (4-mm diameter) was made in the centre of the plate using sterile cork borer, filled with 10 µl of the bacterial supernatant and incubated at 37˚C for 48 hrs. the developed clear zones around the wells were measured (mm) and the data of two biological and six technical replicates were analysed using GraphPad Prism version 7 (GraphPad Software, California, USA).

5.3 Results

5.3.1 Colony variants

The original progenitor strain of *P. aeruginosa* used to generate biofilms was 3.084 ±0.248mm diameter and formed smooth circular colonies with a yellow pigment. Serial dilution and viable plating of the control (BC5) and honey passaged (BP5) *P. aeruginosa* biofilms onto Mueller Hinton agar plates yielded four colony morphotypes as follows: Honey adapted colony variant 1 (BP5 V1), were larger than the parent strain with an average diameter of 3.65 mm ± 0.371 (P<0.05) but was similar in morphology to the parent strain (smooth circular with yellow pigmented colonies); BP5 V2, were significantly smaller than the parent strain with a mean diameter of 1.074 mm ± 0.038 (P<0.0001), yellow to green pigmented,

circular smooth colonies. In passage control experiments a further two morphotypes were observed: BC5 V1, comparable size and colour as the parent strain with an average diameter of 3.214 mm ± 0.103 (P= 0.313), with yellow pigmentation; BC5 V2 were small in size with a mean diameter of 1.477mm ± 0.088 (P<0.0001) with similar shape and colour to BP5 V2. An overview of morphotype appearance and size is shown in Figures 5.1.

The parent strain of *S. epidermidis* used to generate biofilms was 1.65± 0.12mm diameter and formed white smooth circular colonies. Passaging and culturing of *S. epidermidis* biofilm biomass with Manuka honey (BP5) showed one colony variant with reduced diameter 0.689mm ±0.044 (P<0.0001) compared to the parent (BP0), with white smooth circular colonies. In passage control strains (BC5), one colony variant was observed and was comparable in diameter (1.4 ±0.062) and overall morphology (white smooth circular colonies) to the parent strain (Figure 5.2).
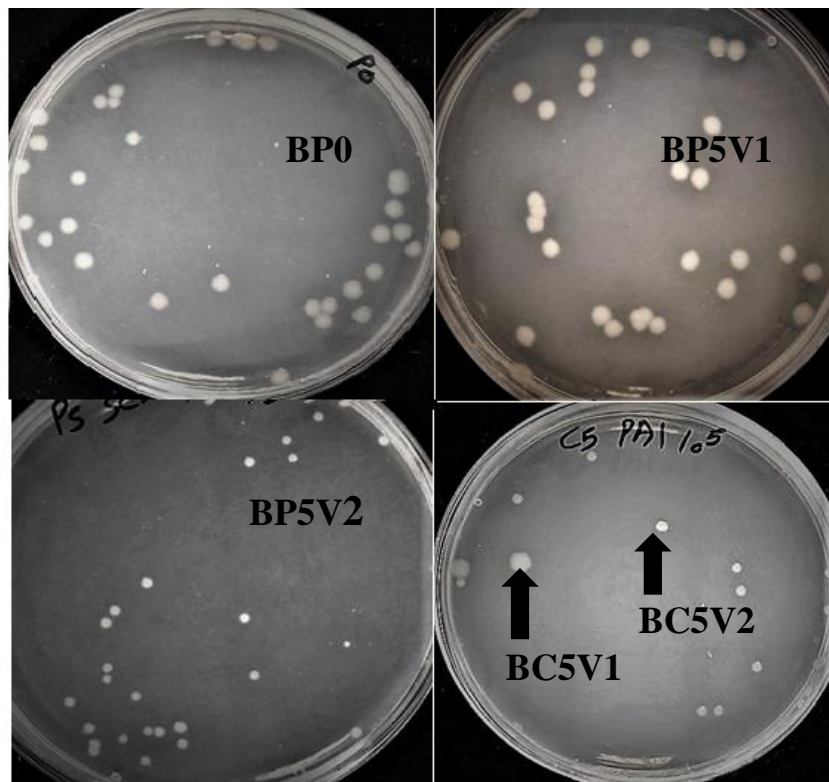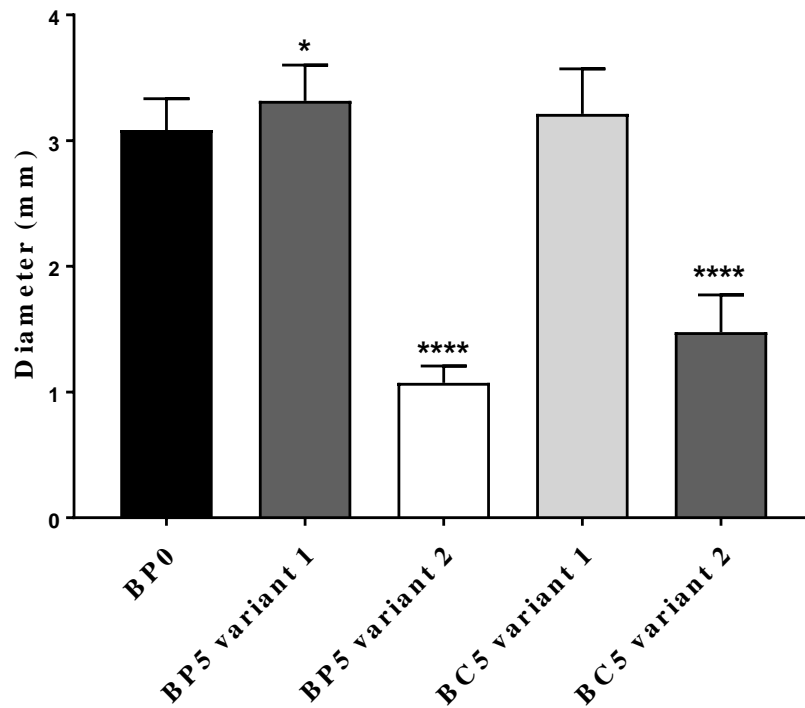
Figure 5. 1 Average colony diameter of *P. aeruginosa* parent strain (BP0), honey-adapted biofilm derived variants (BP5 variant 1 and BP5 variant 2) and honey-free biofilm derived variants (BC5 variant 1 and 2) measured using ImageJ software. Error bars indicate standard deviation (n=12 Biological and technical replicates).
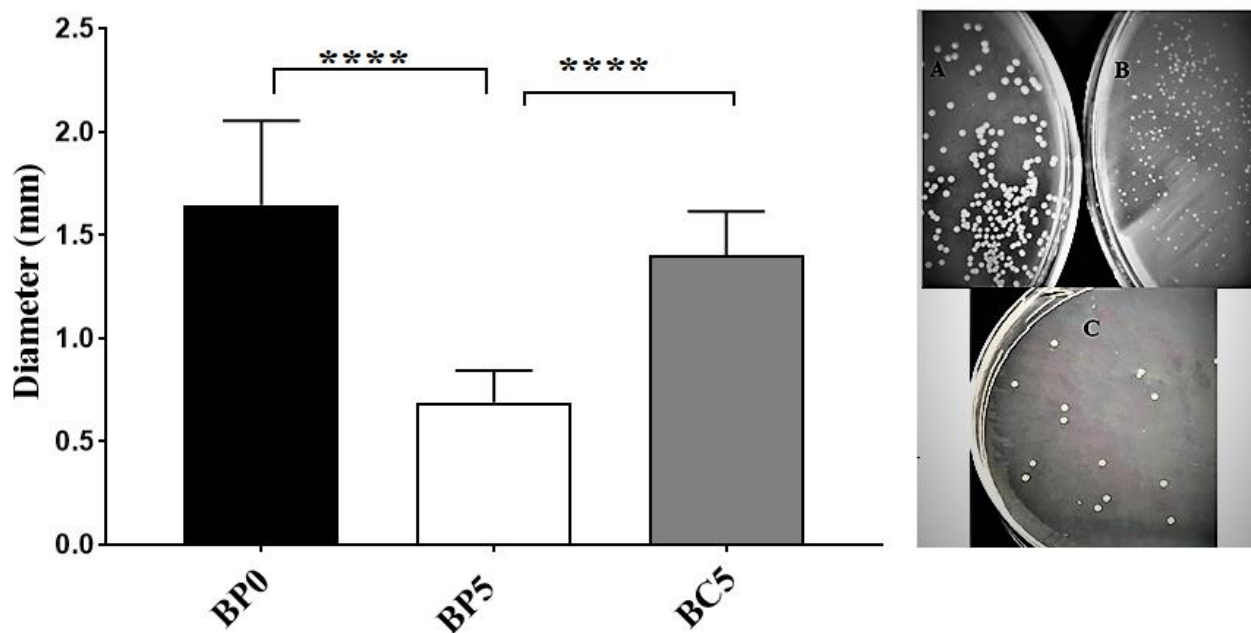
Figure 5. 2 Colony size of BP0, BP5 and BC5 of *S. epidermidis* strain. BP5 showed reduced colony size compared to BP0 and BC5, A) parent strain; B) honey-adapted strain (BP5); C) control strain (BC5). ****Significant change (p <0.0001). Results are mean and standard deviation from two separate experiments (n=12).

5.3.2 Antimicrobial susceptibility testing

MBECs were determined for all colony variants following five passages in the presence of honey (BP5), Mueller Hinton broth only (BC5) and were compared to biofilms generated from the non-passaged parent strain (BP0). The honey adapted colony variant 1 (BP5 V1) exhibited an MBEC of 52.5 %w/v (P>0.05), while the honey adapted colony variant 2 (BP5 V2) showed an MBEC level of 76.25%w/v (P<0.0001) when compared to the *P. aeruginosa* parent strain biofilms (MBEC 40% w/v). The control colony variant 1 (BC5 V1) and the control colony variant 2 (BC5 V2) showed an MBEC of 45%w/v (P>0.05) and 57.5%w/v (P<0.01), respectively (Table 5.1). The culture explanted from *S. epidermidis* honey exposed biofilms

(BP5) showed an MBEC of 60% w/v, an observation significantly greater (P<0.017) than values determined for parent (30% w/v) and control derived samples (BC5) (30% w/v) (Table 5.2).

Regarding antibiotic sensitivities, isolates generated from Manuka honey exposed *P. aeruginosa* biofilms (BP5 V2) showed a reduction in sensitivity to both ciprofloxacin and gentamicin. The MBEC for ciprofloxacin increased 4-fold for BP5 V2 (=31.25 mg/l) when compared to parent (5.85 mg/l) and control strains (BC5 V1= 5.368 mg/l, BC5 V2= 7.812 mg/l). In addition, honey adapted biofilms of *P. aeruginosa* (BP5 V2) exhibited a reduction in sensitivity to gentamicin by 6-fold (890.8 mg/l) in comparison to parent strain (125mg/l) and the control colony variants (BC5 V1=109.4mg/l, BC5 V2=93.75mg/l) (Table 5.1). It can be seen from the data in table 5.2, that honey passaged *S. epidermidis* biofilms (BP5) showed a decrease in susceptibility to vancomycin (500 mg/l, 7-fold) when compared to both the original parent (BP0) (62.5 mg/l) and the control derived cultures (BC5) (62.5mg/l) after the fifth passage with Manuka honey.

Table 5. 1 MBECs of *P. aeruginosa* biofilm.

| Antimicrobial | MBEC | | | | |
	BP0	BP5 variant 1	BP5 variant 2	BC5 variant 1	BC5 variant 2
Medihoney (%w/v)	40%	52.5 % (9.57)	76.7% (5.2)	45% (10)	57.5% (5)
Ciprofloxacin (mg/l)	5.85 (2.26)	15.6	**31.25**	5.368 (2.93)	7.812
Gentamicin (mg/l)	125	125	**890.8 (547.7)**	109.4 (31.25)	93.75 (36.08)
Meropenem (mg/l)	31.25	23.42 (9.04)	31.25	31.25	27.33 (7.82)

Minimum biofilm eradication concentrations before exposure to honey (BP0), after 5 passages in the presence of honey (BP5 variant 1 and variant 2), and after 5 passages in a honey-free media (BC5 variant 1 and variant 2). Data represent the geometric mean taken from two separate experiments each with three technical replicates. Standard deviations are given in parenthesis.

Table 5. 2 MBECs for *S. epidermidis* biofilm.

| Antimicrobial | MBEC | | |
	BP0	BP5	BC5
Medihoney (% w/v)	30	60	30
Ciprofloxacin (mg/l)	7.812	15.6	7.812
Vancomycin (mg/l)	62.5	**500**	62.5

Minimum biofilm eradication concentrations before exposure to honey (BP0), after 5 passages in the presence of honey (BP5), and after 5 passages in a honey-free media (BC5). Data represent the geometric mean taken from two separate experiments each with three technical replicates.

5.3.3 Determination of biofilm formation

Biofilm growth was determined using a crystal violet assay for parent, biofilm derived passaged strains and control strains. BP5 V2 and BC5 V2 of *P. aeruginosa* exhibited a significant (P< 0.001) increase in biofilm formation ability compared to BP0. Also, honey derived biofilm strain of *S. epidermidis* (BP5) showed an increase in biofilm formation compared to parent (BP0) and control (BC5) strains (Figure 5.3). This increase in biofilm formation ability was statistically significant (P< 0.05).
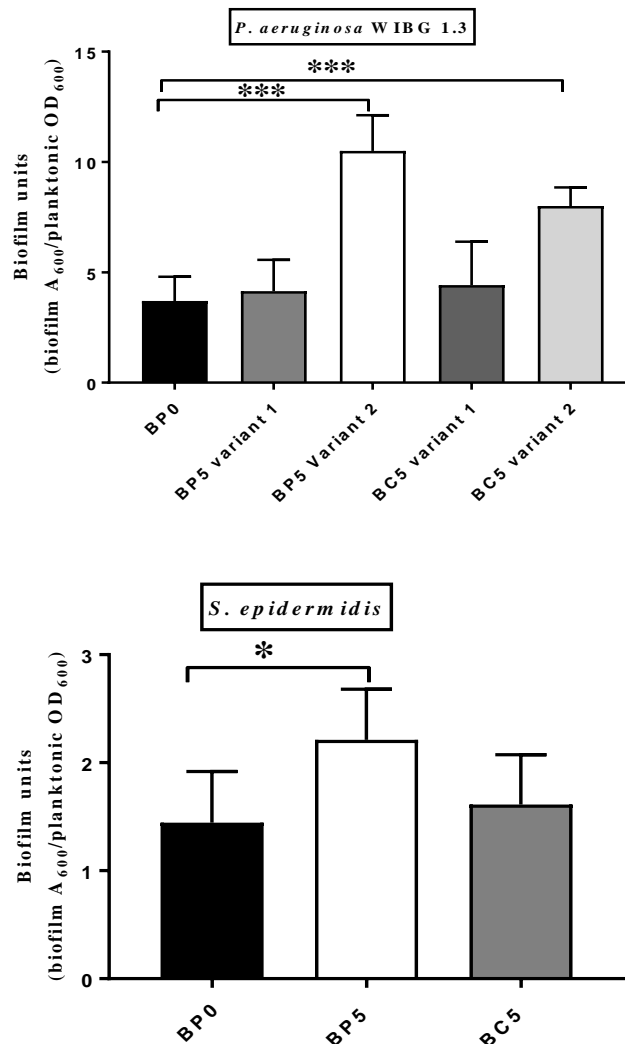
Figure 5. 3 Biofilm formation in honey-adapted strains (BP5), parent (BP0) and control (BC5) strains following adjustment for planktonic mass. Significance was determined following pairwise comparison with parent strains and donated as * and *** (P ≤ 0.05 and 0.001 , respectively).

5.3.4 Determination of growth rate in strains isolated from passaged biofilm

P. aeruginosa honey adapted biofilm variant 2 (BP5 V2) exhibited a significant decrease in growth rate and generation time after five passages with Manuka honey wound gel (Table 5.3). No significant changes in growth curve metrics were observed in *P. aeruginosa* biofilm variant 1 (BP5) and the control biofilm variants (BC5 V1 and BC5 V2). In addition, *S. epidermidis* honey-adapted biofilm variant (BP5) showed a significant decrease in growth rate and carrying capacity while the generation time was significantly increased after five passages with Manuka honey in comparison with the parent and the control strains (Table 5.3).

Table 5. 3 Growth curve metrics generated in parent, control and honey passaged strains of bacteria

Bacteria	Strain	Carrying capacity (k)	Growth rate (h^{-1})	Doubling time (h^{-1})
P. aeruginosa WIBG 1.3	BP0	0.560 (0.190)	0.673 (0.279)	1.198 (0.480)
	BP5 variant 1	0.610 (0.136)	1.662 (0.743)	0.743 (0.468)
	BP5 variant 2	0.568 (0.176)	1.227 (0.169)**	0.555 (0.068)**
	BC5 variant 1	0.438 (0.027)	0.714 (0.137)	1.004 (0.194)
	BC5 variant 2	0.403 (0.121)	0.530 (0.125)	1.364 (0.278)
S. epidermidis	BP0	0.2955 (02955)	0.2921 (0.0533)	2.438 (0.4213)
	BP5	0.2052 (0.0134)**	0.2246 (0.0434)*	3.178 (0.5539)*
	BC5	0.2535 (0.0529)*	0.2660 (0.0306)	2.643 (0.367)

Significance denoted as $P \leq 0.05$ (*) or $P \leq 0.01$ (**) following pairwise comparison to baseline data (BP0). Standard deviations are given in parenthesis.

5.3.5 Gene mutations in both *P. aeruginosa* and *S. epidermidis.*

Table 5.4 shows all identified mutations in biofilm-derived variants of *P. aeruginosa* when compared to the original progenitor strain. BP5V2 showed a missense mutation within three different coding genes that were absent in the control variants, including ubiD-like decarboxylase, protein-glutamate methylesterase and transcriptional activator protein lasR. These mutations resulted in various amino acid sequences and the mutations within the Protein-glutamate was a single nucleotide substitution (G to A) resulting in an early stop codon. Both small variants of the honey-adapted (BP5V2) and passage controls (BC5V2) exhibited missense mutations within cytochrome C1 (fbcH) and methyl-accepting chemotaxis protein (cheB-1) genes. Point mutations within the phzF-2 and nqrD genes were observed in the passage control derived cultures but not in the honey-adapted variants.

It can be seen from the data in table 5.5 that missense mutations in four variant genes including sdrG, cdaR, scrK and lipA were seen in the BP5 sequence of *S. epidermidis*. Three of these genes (cdaR, scrK and lipA) were shown to be involved in carbohydrate, sugar, and lipid metabolism, whilst the sdrG gene has an important role in cell adhesion. Such mutations were absent from the control passages.

Table 5. 4 Summary mutations and resulting amino acid changes in *P. aeruginosa* WIBG 1.3 after honey exposure.

Bacterium	Gene	Protein encoded	BP5 (Variant 1)	BP5 (Variant 2)	BC5 (Variant 1)	BC5 (Variant 2)
			LCV	SCV	LCV	SCV
***P. aeruginosa* WIBG 1.3**	fbcH	Cytochrome C1	-	V81M	-	V81M
	cheB_1	Protein-glutamate methylesterase	-	Q124*	-	-
	mcpB	Methyl-accepting chemotaxis protein	-	T216A	-	T216A
	hudA	UbiD-like decarboxylase	-	G312S	-	-
	lasR	Transcriptional activator protein lasR	-	S223F	-	-
	phzF-2	Trans-2,3-dihydro-3-hydroxyanthranikate isomerase	-	-	T261A	T261A
	nqrD	Na+ translocating NADH-quinone reductase subunit D	-	-	-	F104V

BP5, following five biofilm passages with honey exposure; BC5, following five biofilm passages in antimicrobial free Muller Hinton broth (control). A: alanine, D: aspartic acid, Q: glutamine, G: glycine, V: valine, M: methionine, T: threonine, S: serine, F: phenylalanine, E: Glutamic acid, L: leucine, I: isoleucine. * means stop codon.

Table 5. 5 Summary mutations and resulting amino acid changes in *S. epidermidis* after honey exposure

Bacterium	Gene	Protein encoded	BP5	BC5
S. epidermidis	sdrG	Serine-aspartate repeat-containing protein G	A31E	-
	cdaR	Carbohydrate diacid regulator	D35V	-
	scrK	Fructokinase	A251V	-
	lipA	Lipase/esterase lipA	L174I	-

BP5, following five biofilm passages with honey exposure; BC5, following five biofilm passages in antimicrobial free Muller Hinton broth (control). A: alanine, D: aspartic acid, Q: glutamine, G: glycine, V: valine, M: methionine, T: threonine, S: serine, F: phenylalanine, E: Glutamic acid, L: leucine, I: isoleucine.

5.3.6 Relative pathogenicity assay.

Increased pathogenicity was observed in both honey-adapted biofilm variants of *P. aeruginosa* (BP5 V1 and BP5 V2) when compared to the parent strain (BP0) and the control variants (BC5 V1 and BC5 V2) (Figure 5.4). In *P. aeruginosa*, 0% of the larvae injected with BP5 variant 1 or variant 2 survived following 7 days incubation, in comparison to 40% of the BP0, 15% of the BC5 V1 and 30% of the BC5 V2 injected wax worms (P<0.0001). Furthermore, the parent (BP0) and the control strains (BC5) of *S. epidermidis* were less virulent than the honey gel exposed strains, and exhibited 60% and 55% survival, respectively, in larvae compared to 20% in the BP5 injected group. These changes in pathogenicity were statistically significant (P<0.05) following pairwise log rank testing.
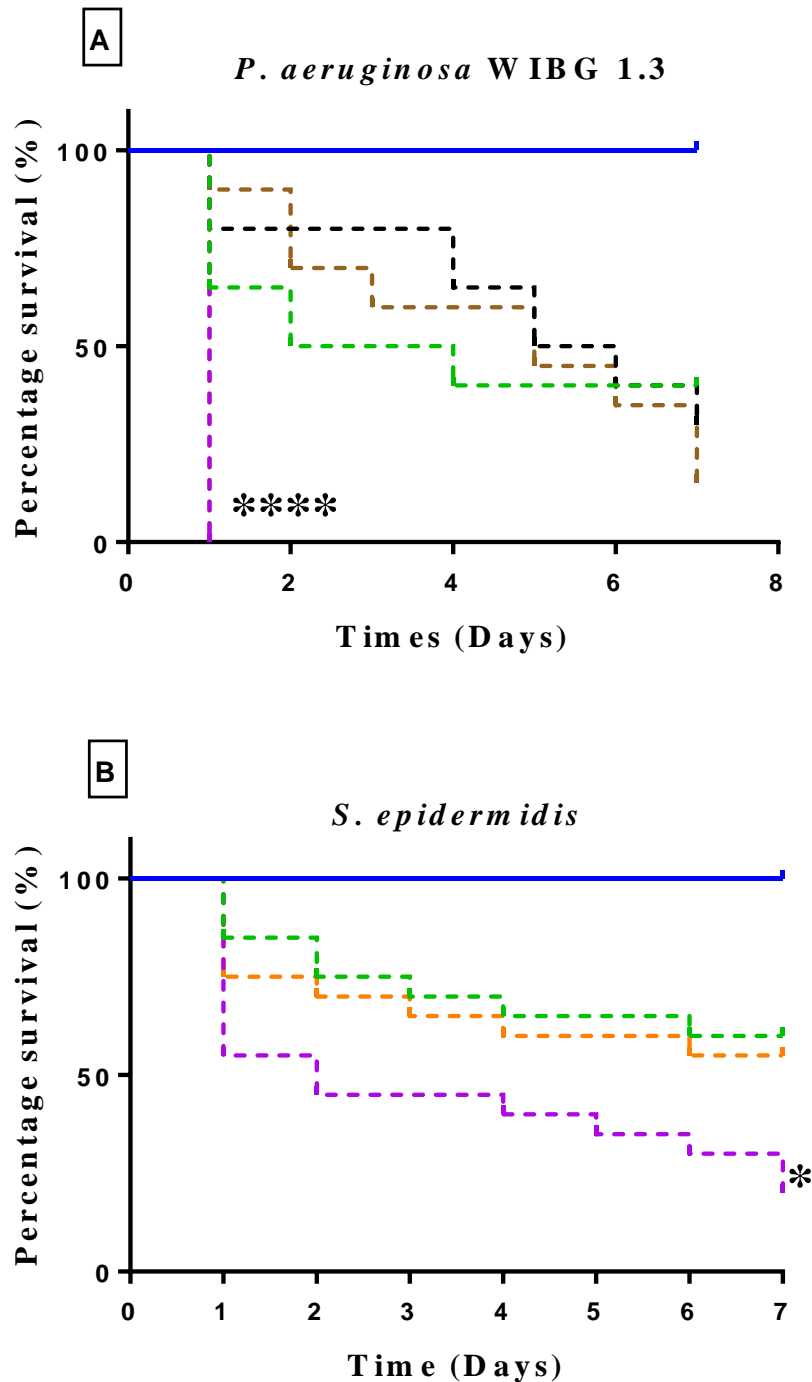
Figure 5. 4 A) Kaplan Meir Curves of *P. aeruginosa* WIBG 1.3 illustrating percentage survival following injection of *Galleria mellonella* (wax moth) with sterile PBS (solid blue line), parent strain (BP0, green dotted line), Medihoney adapted BP5 variant 1 and 2 (purple dotted line), BC5 variant 1 (brown dotted line) and BC5 variant 2 (black dotted line). B) Kaplan Meir Curves of *S. epidermidis;* sterile PBS (solid blue line), parent strain (green dotted line), BP5 (Purple dotted line) and BC5 (orange dotted line). Significance in virulence between BP0 vs BP5 and BP5 vs BC5 were determined using log-rank testing with significance denoted as P < 0.05 (*) or P < 0.0001 (****).

5.3.7 Evaluation of pyocyanin level in *P. aeruginosa* WIBG 1.3

Biofilm variant 2, isolated from wound gel passaged *P. aeruginosa* biofilms, exhibited green pigmentation when grown on both solid agar and in broth. Pyocyanin production was therefore determined and compared amongst parent strain, honey passaged and the control biofilm derived variants using a chloroform extraction methodology. Honey adapted colony variant 2 (BP5V2) showed a highly significant increase in the production of pyocyanin when compared to the parent (P0 vs BP5 V2, 354.2% ± 61.5; P=0.0002) and the control passaging variants (BC5V1=124% ±24.45; P<0.0001, BC5V2=75.48%±32.88; P<0.0001). No significant changes in pyocyanin production were observed in the other colony variants (BP5V1, BC5V1 and BC5V2)(Figure 5.5).
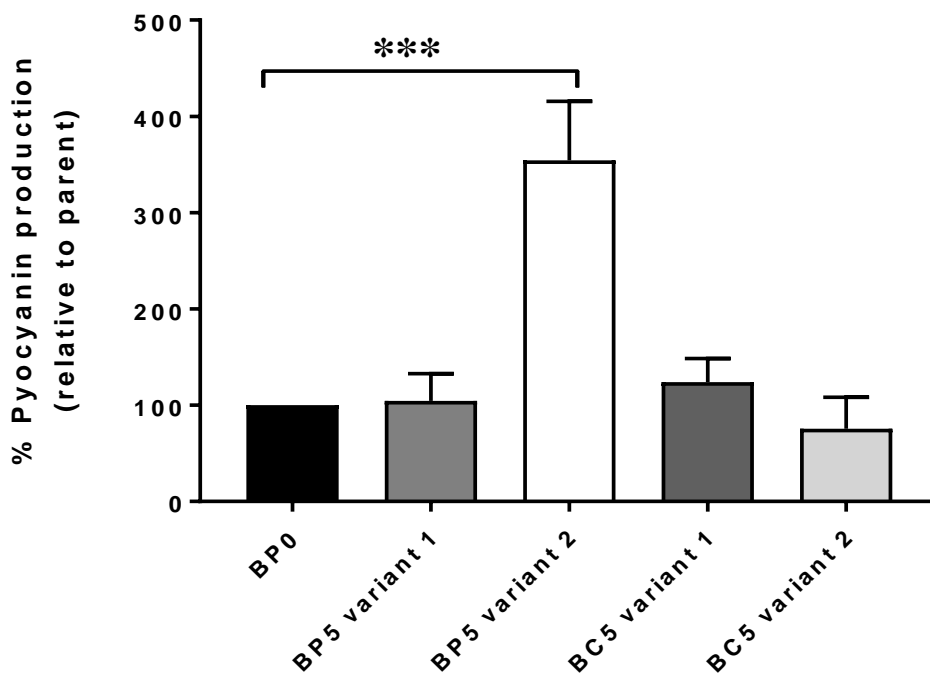
Figure 5. 5 Pyocyanin production by *P. aeruginosa* WIBG 1.3 biofilm strains. parent strain (BP0, black), Medihoney-adapted variant 1 (BP5, grey), Medihoney-adapted variant 2 (BP5, white), the control variant 1 (BC5, dark grey) and the control variant 2 (BC5, light grey) bacteria. Significant data are represented as *** (P =0.0002). Error bars represent standard deviation (n=6).

5.3.8 Swimming, swarming and twitching motility in *P. aeruginosa* WIBG 1.3

On swarming agar plates, both small variants of the honey-adapted (BP5V2) and the control strain (BC5V2) of *P. aeruginosa* showed a significant decrease in motility in comparison to the parent, BP5 variant 1 and BC5 variant 1 ($P<0.0001$) (Figure 5.6, B). Furthermore, the smaller honey exposed variant (BP5V2) showed a significant decrease in twitching motility ($P< 0.05$) when compared to the parent and the control strains (Figure 5.6, C). However, no significant difference was observed on swimming plates between wild type and passaged bacteria (Figure 5.6, A).
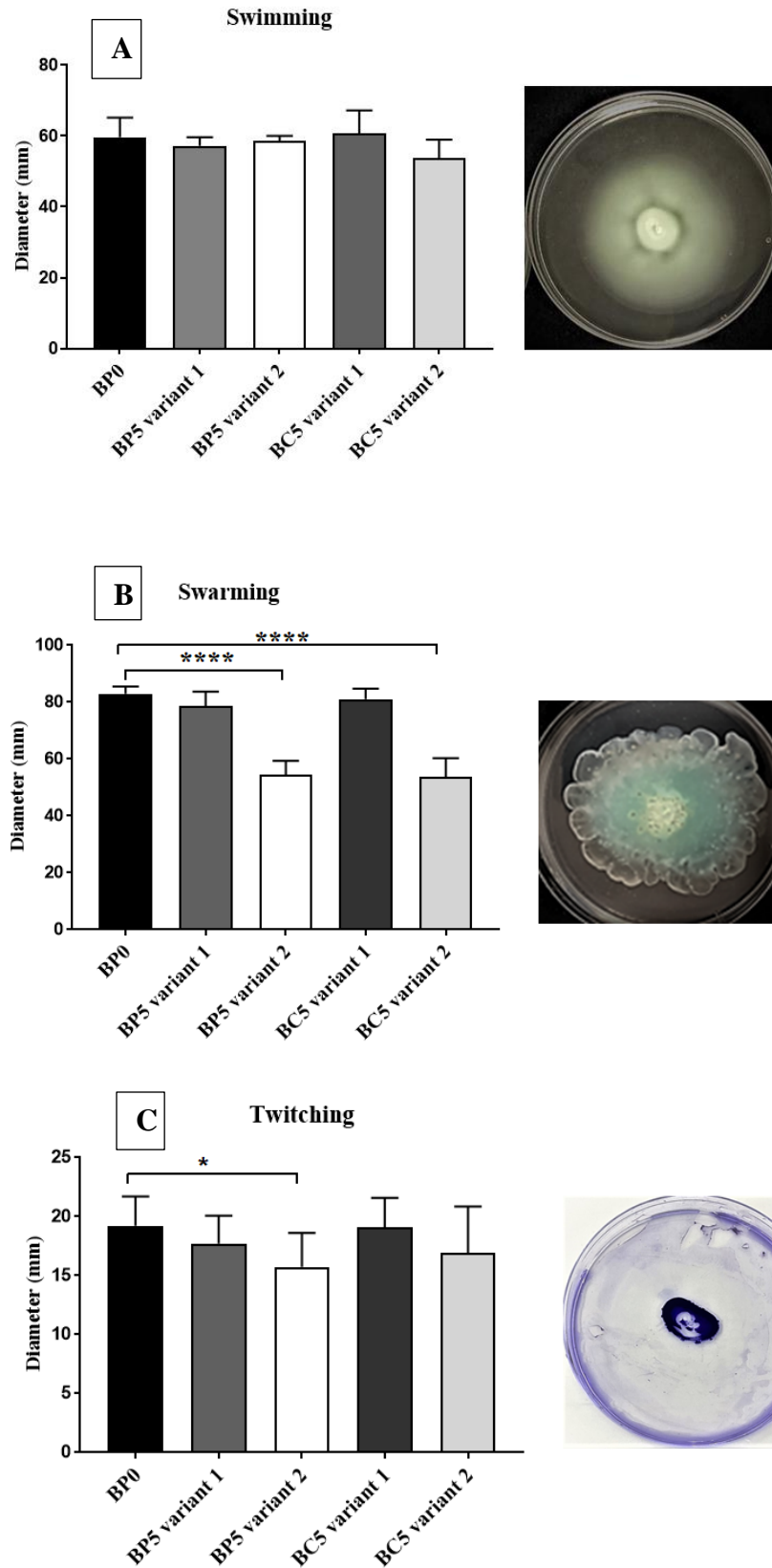
Figure 5. 6 Effect of Manuka honey on *P. aeruginosa* WIBG1.3 biofilm strains swimming (A), swarming (B), and twitching (C) motilities. Values shown represent the mean diameter of corresponding motility zones and error bars represent the standard deviation (SD) of two representative experiments, with triplicate plates per experiment.

5.3.9 Protease and elastase activities in *P. aeruginosa* biofilm strains.

Protease and elastase activities of *P. aeruginosa* biofilm strains were assessed using azocasein and congo red assay. As shown in Figure 5.7, a significant increase in the production of protease was observed in BP5 colony variant 2 when compared to variant 1, the control colony variants (BC5 V1 and V2) and their unexposed counterparts (P<0.05). Additionally, honey passaged bacteria (BP5 variant 2) showed an increase in elastase production (P<0.01) compared to BP0, both variants of BC5 and BP5 variant 1 biofilm strains (Figure 5.8).
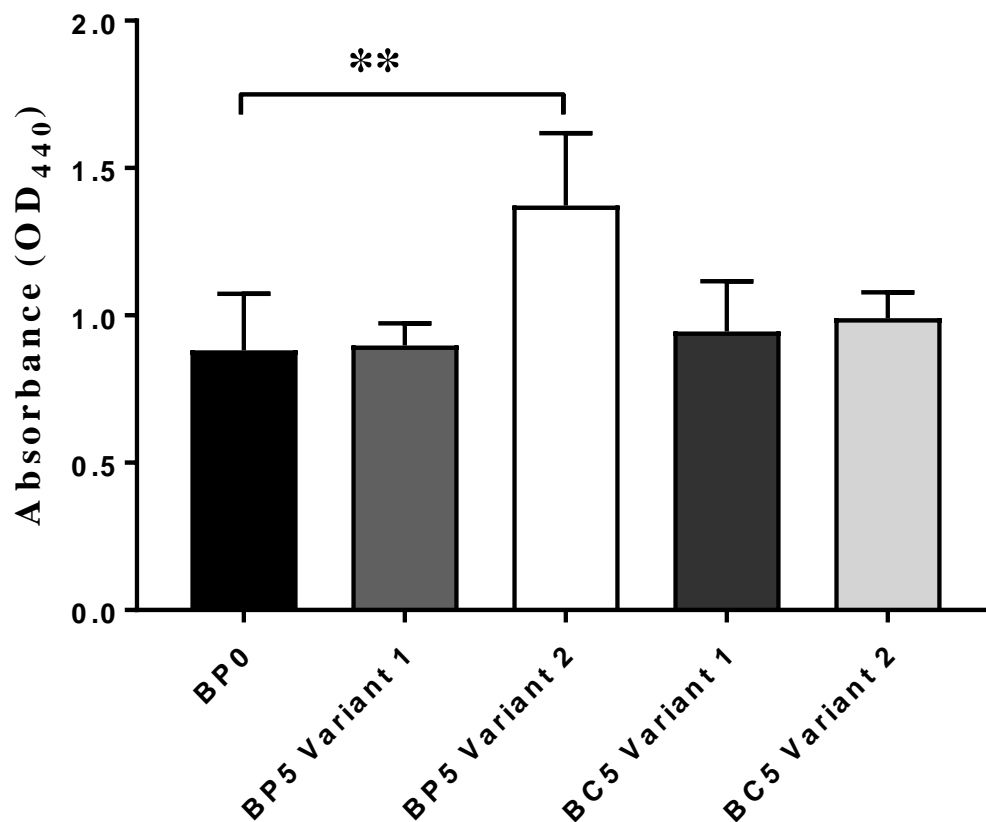
Figure 5. 7 Extracellular proteolytic activity (azocasein assay) of *P. aeruginosa* WIBG 1.3 before (BP0), after five passages with Manuka honey (BP5 variant 1 and 2), and after five passages in honey-free environment (BC5 variant 1 and 2). Data represent the mean of two biological and eight technical replicates. Error bars indicate standard deviation. Significant differences in protease levels are indicated as ** (P=0.003).
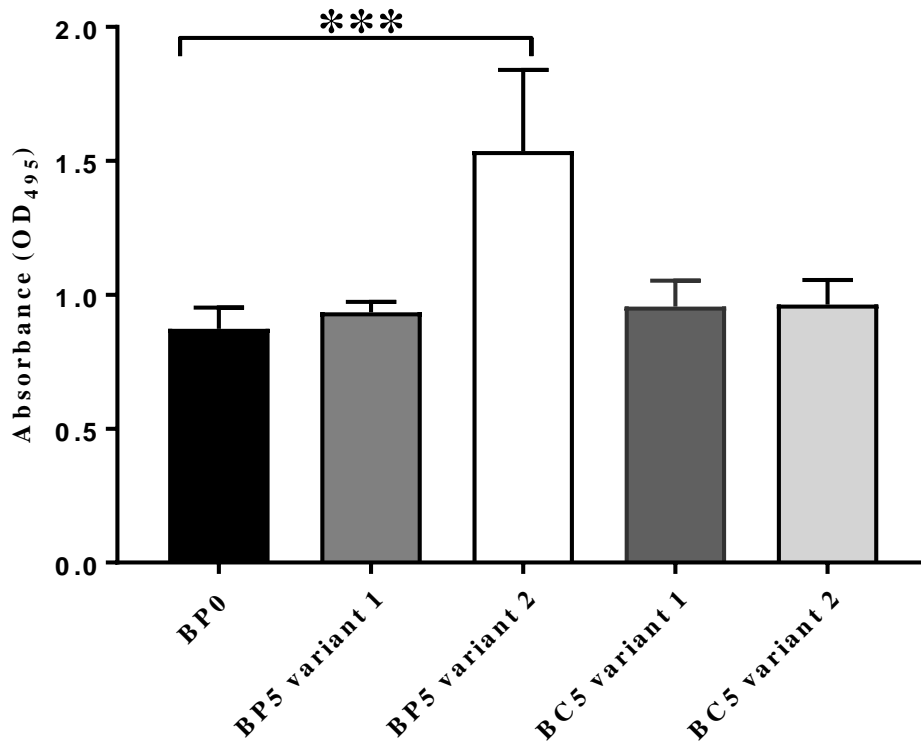
Figure 5. 8 Elastase assay of *P. aeruginosa* WIBG 1.3 biofilm strains: breakdown of insoluble elastin-Congo Red (OD_{495}), with two biological and eight technical replicates. P values were considered significant when compared with the parent (BP0) and the control (BC5), *** ($P=0.0003$).

5.3.10 Reduced production of extracellular lipase by *S. epidermidis* after Manuka honey exposure

Lipase producer strains were identified using Rhodamine-B plates containing olive oil. The formation of orange fluorescent colonies or halos surrounding the colonies when exposed to ultraviolet light was considered as a positive result. All parent and passaged *S. epidermidis* were categorized as lipase producing bacteria. These strains were subjected to rapid quantification screening using Rhodamine B-olive oil plates by measuring the zones of intensification around the wells. Honey adapted biofilm strains (BP5) showed a decrease in lipase production ($P<0.05$) when compared to parent and control strains (Figure 5.9). In order to support the results of Rhodamine B-olive oil plates, the clear zones around the wells of BP5

strain in Tween80 agar plates were measured and compared to BP0 and BC5 strains. As shown in figure 5.10, the smaller, passaged variant (BP5) displayed a reduction in extracellular lipase production when compared to BP0 (P<0.01) and BC5 (P<0.05).

lipase (olive oil+rhodamine b plat)
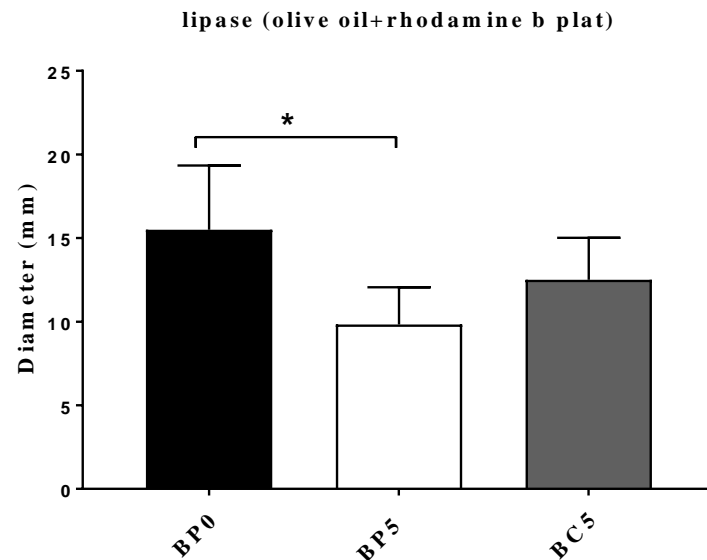
Figure 5. 9 The diameter of intensification (Orange) zone of lipase producing *S. epidermidis* biofilm strains on Rhodamine B-olive oil plates. BP0= Parent strain, BP5= Manuka honey adapted strain (SCV) and BC5= Control (exposed to honey-free medium). Error bars show standard deviation (n=6). Significant differences are indicated as * (P<0.05).

Lipase (Tween 80 without indicator)
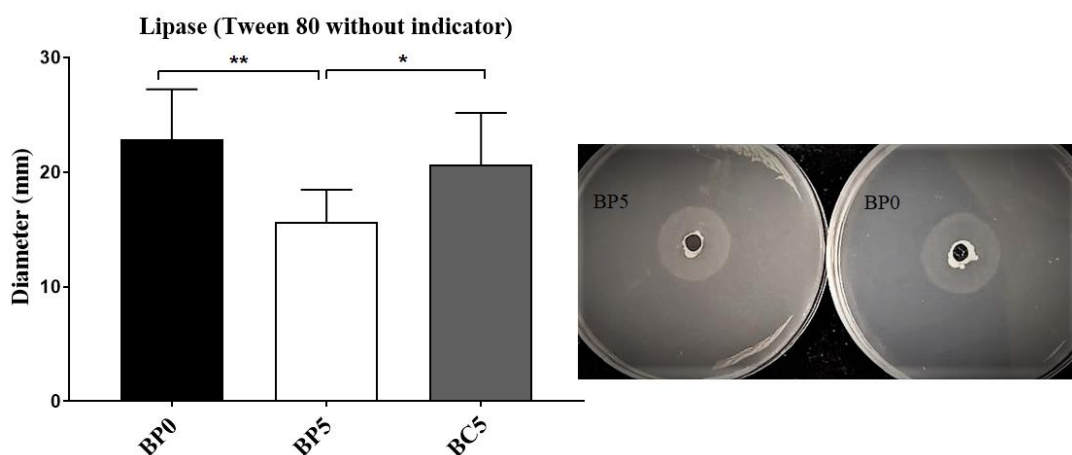
Figure 5. 10 The diameter of clear (WHITE) zone of *S. epidermidis* parent strain (BP0), after 5 passages with Manuka honey (BP5) and after 5 passages in MH broth as a control (BC5) on Tween80 plates. Data represent the mean of two biological and six technical replicates. Error bars show the standard deviation (n=6). Significant differences in lipase activity were indicated as * (P<0.05); ** (P<0.01).

5.4 Discussion

A biofilm is a complex environment of micro-organisms in which microbial cells attach to each other on a living or non-living surface within a self-formed extracellular polymeric matrix (Høiby et al., 2010, Oates et al., 2014). Several studies have demonstrated the existence of bacterial biofilms in chronic wounds (Malone et al., 2017, Oates et al., 2014, James et al., 2008). Biofilm associated infections are by nature, difficult to treat due to antimicrobial recalcitrance (Ceri et al., 1999, Amorena et al., 1999). The increased antibiotic resistance of biofilms was due to the existence of exopolysaccharide matrix, decreased growth rate, biofilm-specific drug-resistant or drug-tolerant physiologies including the presence of persisters cells and small-colony variants (SCVs) (Stewart and Costerton, 2001, Gilbert et al., 2002a, Mah and O'Toole, 2001). Various phenotypic and genetic differences have been discovered in isolates obtained from the biofilm during chronic infections (Allegrucci and Sauer, 2008, Kirisits et al., 2005, Darch et al., 2015). For instance, adapted morphotypes, mucoid and small colony variant, of *P. aeruginosa* have been found in cystic fibrosis samples (Sousa and Pereira, 2014, Lozano et al., 2018). Biofilms play a significant role in wound chronicity; hence, the application of antimicrobial products are often required for sustained periods of time. The main aim of the current investigation was to study the phenotypic and genomic changes underlying chronic Manuka honey exposure on biofilm phenotypes through laboratory passaging experiments using a clinical grade wound gel.

Camplin and Maddocks (2014) reported that *P. aeruginosa* biofilms isolates exposed to sublethal concentrations of Manuka honey developed slow growing isolates with reduced sensitivity to Manuka honey, imipenem and rifampicin as well as enhanced biofilm forming capacity (Camplin and Maddocks, 2014). In the present study, a marginal decrease in Manuka honey sensitivity was noted in both *S. epidermidis* and *P. aeruginosa* following five passages. It is interesting to note that in passaged, biofilm derived bacteria, colonies of reduced diameter

were noted and subsequently shown to exhibit a ≥4-fold reduction in sensitivity to ciprofloxacin and gentamicin in pseudomonas, and to vancomycin in *S. epidermidis* when compared to baseline and control datasets. Such changes in susceptibility profiles are supported by our previous investigations with planktonic isolates (Mokhtar et al., 2020).

Small colony variants are typically characterised as slow growing bacteria that develop spontaneously within microbial populations in response to environmental stresses, such as antimicrobial treatment, (Johns et al., 2015, Wright et al., 2013) and may show transient or sustained phenotypic variations (Camplin and Maddocks, 2014). Clinically, the presence of SCVs during infection is often correlated with recurrent or chronic infectious disease (Johns et al., 2015). Exposure to sub-therapeutic concentrations of triclosan has been previously observed to induce SCV formation (Bayston et al., 2007). Triclosan-induced small colony variants of *S. aureus* have been associated with reduced triclosan susceptibility, decreased competitive fitness (Forbes et al., 2015) and attenuated virulence (Latimer et al., 2012). The tendency for SCVs to resist antimicrobial treatment is attributed to their slow growth, impaired electron transport chains (Lannergård et al., 2008), and enhanced biofilm formation (Proctor et al., 1998), which was observed in this study and may explain the increase in MBEC towards Manuka honey in passaged cultures. Small colony variants differed from the normal phenotype not only in their small colony size, but also decreased growth rate, lack of pigmentation, reduced haemolytic activity and altered expression of virulence factors (Proctor et al., 1998, Acar et al., 1978, Proctor et al., 2006, McNamara and Proctor, 2000). In this study, colonies with reduced diameter demonstrated a reduction in the growth rate associated with increased production of pyocyanin, protease and elastase in *P. aeruginosa* and decreased lipase production in *S. epidermidis*. It is unclear if SCV formation was triggered by the continued exposure to honey or as a result of culture based passaging on laboratory media as a colony of reduced diameter was also noted in control exposures, although it must be noted that changes

in antibiotic susceptibility, virulence, growth rate, pyocyanin, protease and elastase were not observed in these isolates.

In an attempt to better understand the underlying mechanisms of observed changes, whole genome sequencing was performed on all biofilm derived variants and compared to progenitor strains. The genome sequencing of pseudomonas honey passage colony variant 2 revealed a nonsynonymous mutation within ubiD-like decarboxylase, protein-glutamate methylesterase and transcriptional activator protein lasR coding genes. However, both small variants of the honey adapted, and the control exhibited a nonsynonymous mutation in cytochrome C (fbcH) and methyl-accepting chemotaxis protein (mcpB-1) genes. Cytochrome C is located on the periplasmic side of the membrane and contains covalently bound heme C and plays an important role in electron transport as well as in the catalysis of numerous redox reactions (Bertini et al., 2006, Simon and Hederstedt, 2011). Previous studies have demonstrated a specific relationship between alterations in electron transport, due to mutations in hemin and menadione biosynthesis genes, and small colony variant formation. Also, mutations in the cytochrome c assembly protein (CtaA) lead to SCVs formation (Proctor et al., 2006, Clements et al., 1999).Therefore, cytochrome c mutation could in part contribute to small colony formations in both the control and honey passaged *P. aeruginosa*.

Swarming motility is a multicellular process involving a coordinated and rapid movement of the bacterial community over a semi-solid surface (Fraser and Hughes, 1999). Swarming is highly dependent on the density of bacterial cells, nutrient media type, and the moisture content of the surface (Wang et al., 2004). The swarming of *P. aeruginosa* has previously been shown to rely on both flagella and type IV pili (Köhler et al., 2000, Overhage et al., 2007). A previous study found that *P. aeruginosa* swarming is a dynamic mechanism of adaptation in response to a viscous environment, resulting in a major shift in the expression of virulence genes such as those encoding type III secretion systems, extracellular proteases, and those responsible for

iron transport (Overhage et al., 2008). In addition, two virulence genes, lasB and pvdQ, were necessary for swarming motility (Overhage et al., 2008). A point mutation in the lasR gene (regulates both lasA and lasB genes) observed as part of the current study could therefore provide a rationale for the decrease in swarming motility observed in the small honey exposed biofilm variant. Swarming and twitching motility also contributes to biofilm development. The magnitude of swarming motility displayed by cells at an early stage in biofilm formation can reflect a juncture at which biofilm production proceeds to form a flat, uniform biofilm or a structured biofilm (Shrout et al., 2006). Twitching motility has also been shown to be essential in the formation of a structured, mature biofilm. Further research investigating such areas would be interesting.

The pathogenicity of *P. aeruginosa* is multifactorial depending on various virulence factors such as secreted and cell-associated factors (Karatuna and Yagci, 2010). In the current study, the increased pathogenicity of honey exposed *P. aeruginosa* was associated with increased biofilm formation and increased production of extracellular protease and elastase. Genome analysis showed a mutation in three virulence-associated genes: hudA (encoding UbiD decarboxylase), lasR (Quorum-sensing regulated gene) and cheB (Protein-glutamate methylesterase). Over expression of the hudA gene has been previously associated with virulence attenuation, as determined using Drosophila melanogaster and mouse models (Kim et al., 2008). Additionally, Howlet et al, 1980, reported that the induction of a functional ubiquinone biosynthesis pathway altered flagellation in *Salmonella typhimurium* (Howlett and Bar-Tana, 1980). Chemotaxis, the directed movement towards chemicals in the environment, has been revealed to play a major role in the pathogenicity of a wide range of bacteria (Matilla and Krell, 2018) such as *P. aeruginosa* (Garvis et al., 2009, McLaughlin et al., 2012, Schwarzer et al., 2016). Mutations in the cheB gene cause a functional defect in clockwise (CW) flagellar rotation although bacteria can still respond to stimuli that enhance counter-clockwise rotation

(CCW) (Parkinson, 1976). Whilst flagellar rotation was not directly investigated in this study, counter-clockwise rotation has been shown to produce smooth swimming which was evidenced by agar based motility assays.

Bacteria use small single molecules to track their population density and organise gene regulation in a process called quorum sensing (QS). Acylated homoserine lactones (AHL) are the most important signal molecules in Gram negative bacteria (Venturi, 2006). There are two AHL QS systems, the las and rhl systems, within *P. aeruginosa* (Venturi, 2006). Both of those systems have been found to control the development of various factors of virulence, including alkaline proteases, elastase, exotoxin A, rhamnolipids, pyocyanin, and biofilm formation (Smith and Iglewski, 2003). The las system, the lasI gene product direct the synthesis of N-(3 oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-AHL), which activates the lasR transcriptional regulator (Venturi, 2006). The lasR gene of *P. aeruginosa* is required for the transcription of two protease genes associated with virulence, elastase (lasB) and protease (lasA) (Gambello et al., 1993). Elastase is a metalloprotease that degrades elastin, collagen, immunoglobulin G, complement and serum protease inhibitors such as α1, α2 and C1 inhibitors. Such activities may exacerbate damage to innate host barriers such as skin and soft tissue, and as a consequence, enhance access of the bacteria to the blood stream (Rumbaugh et al., 1999). Protease inactivates essential host defence proteins such as complement, cytokines and antibodies by interfering with fibrin formation (Smith et al., 2006b). Manuka honey has previously been shown to down-regulate three genes necessary for functional quorum sensing in methicillin resistant *Staphylococcus aureus*, with knock-on effects on virulence and biofilm genes (Jenkins et al., 2014).

Staphylococcus epidermidis is a coagulase negative staphylococci that is frequently isolated from wounds, but healing was carried out without infection or inflammation. Coagulase negative staphylococci were later revised to be of significance in wounds from unimportant

skin flora into opportunistic pathogens, and their existence in biofilm was connected to delayed, recurrent and persistent infections related to indwelling medical devices (Smith and Hogan, 1995). Generally, the success of *S. epidermidis* as a pathogen must be due to its ability to adhere to surfaces and to stay there under the protection of extracellular protective material (Vuong and Otto, 2002). *S. epidermidis* in this study showed an increase in virulence after biofilm honey exposure compared to parent and control strains, associated with increased ability to form biofilm, and decreased lipase production. Such increases in virulence and biofilm formation after ten passages with Manuka honey wound gel were previously observed in *S. epidermidis* and *P. aeruginosa* when grown planktonically (Mokhtar et al., 2020). In *S. epidermidis* infections, biofilm formation is considered to be the major virulence factor (Raad et al., 1998). At the genomic level, a nonsynonymous sdrG (A31E) mutation was identified in *S. epidermidis* after five biofilm passages with Manuka honey (BP5). Fbe (sdrG) proteins of *S. epidermidis* are surface proteins, which show similarity to *S. aureus* fibrinogen receptor or clumping factor (ClfA) (Vuong and Otto, 2002), promote adherence of bacteria to immobilized fibrinogen substrates. SdrG-mediated binding to fibrinogen is essential for staphylococci to interact with an indwelling device and initiate biofilm formation (Sellman et al., 2008). Incubation of *S. epidermidis* with anti-SdrG immune serum has been shown to reduce the bacteria retrieved in a mouse model of infection (Rennermalm et al., 2004).

Genomic data revealed a mutation in the cdaR gene of isolate BP5 of *S. epidermidis*, an observation that was notably absent from controls. CdaR is a regulator controlling cdaA (Cyclic-di-AMP synthase) activity in *Bacillus subtilis* (Mehne et al., 2013a) and *Listeria monocytogenes* (Rismondo et al., 2016). It has been shown that c-di-AMP is essential for the integrity of the cell wall in numerous Gram-positive bacteria (Mehne et al., 2013a, Corrigan et al., 2011, Rismondo et al., 2016). Furthermore, resistance to cell wall-targeting antibiotics can occur as a consequence of alterations in the intracellular c-di-AMP pathway. Rismondo et al,

2016, observed that penicillin had no effect on the killing of cdaA depleted cells (Rismondo et al., 2016). Interestingly, the lack of cdaR increases bacterial resistance to lysozyme (Rismondo et al., 2016). As such, cdaR mutation in *S. epidermidis* BP5 may contribute to the reduced sensitivity observed with regards to vancomycin in honey treated *S. epidermidis* biofilms.

In the current study, *S. epidermidis* exhibited a decrease in lipase production after five biofilm passages with Manuka honey on both olive oil and Tween80 substrate plates despite a concomitant increase in *Galleria* virulence. Whole genome sequencing showed a missense mutation in the lipA gene in the BP5 sequence of *S. epidermidis*. Lipases have been implicated as a possible virulence factor in a number of skin diseases such as boils or abscesses (Hedström, 1975, Rollof et al., 1987). An experiment using *in vitro* expression technology (IVET) demonstrated that lipases are produced in a murine abscess model infection (Lowe et al., 1998). Additionally, *Staphylococcus* spp produce lipoic acid via LipA that contribute to the capability of these microorganisms to colonize host tissues (Zorzoli et al., 2016). A previous study found that mice infected with a lipA mutant of *S. aureus* demosntrated the ability to clear infections more than mice infected with the wild-type *S. aureus* (Grayczyk et al., 2017). This improvement in the clearance of bacteria may be due to growth deficiencies as a consequence of lipoic acid limitation or due to activation of macrophages (Grayczyk et al., 2017). Although it has been indicated that lipases may be crucial for the colonization and persistence of resident bacteria on the skin, the role of these enzymes in virulence is not clearly understood (Gribbon et al., 1993).

Taken together, these results suggest that the biofilm mode of growth of *P. aeruginosa* and *S. epidermidis* in the presence of Manuka honey wound gel may quickly select for biofilm-specific variants that show reduced sensitivity to Manuka honey and certain antibiotics. Biofilm-colony variants with reduced colony diameter exhibited phenotypic changes such as biofilm formation ability, pathogenicity, motility, and exotoxin production. These phenotypic

variations were associated with multiple genomic mutations that may explain some of the changes that were observed in this study. The clinical implication of such adaptations to Manuka honey in chronic wound management are unclear. Whilst bacteria remained sensitive to in use concentrations of wound gel, changes in virulence could impact upon wound healing and as such, need further investigation.

Chapter 6

General Discussion

6.1 Overview

Chronic wounds represent a significant burden to both the patient and the medical system (Han and Ceilley, 2017). Infection is the most common preventable challenge to wound healing, and topical antimicrobials have been used empirically to prevent wound infection. While bacteria are a natural part of the skin microbiota, and therefore wounds, a crucial threshold of 10^5 bacteria has been suggested as a distinction between colonisation and a clinically significant infection that may hinder wound healing (Trengove et al., 1996). Numerous antimicrobial dressings, including Manuka honey impregnated dressings, are available for the potential prevention and/or treatment of wound infections (Zone and Guide, 2017). These topical antimicrobials exhibit activity towards a broad spectrum of microorganisms and usually have several pharmacological targets (Bradshaw, 2011).

In various parts of the world, honey has been licenced for topical use, such as in wound healing (Carter et al., 2016b). In England, prescription costs for advanced and antimicrobial wound dressings are over 110 million pounds per year. Despite this, scientific evidence supporting the use of antimicrobial wound dressings for the treatment of wound infections, however, is less well known and of lower quality than in many other prescription field (NICE, 2016). Honey is normally inexpensive and not harmful to patients (Dunford and Hanano, 2004), prevents bacterial growth and can facilitate wound healing (Molan, 1999, Carter et al., 2016b). Despite these advantages, a number of open questions remain as to whether bacterial exposure to sub-inhibitory concentrations of honey results in changes in antimicrobial sensitivity or phenotypic adaptation associated with attenuated/enhanced virulence potential. Manuka honey dressings are often used for long periods of time and can result in viable cells that are exposed to sub-therapeutic concentrations of honey due to contact with serum and wound fluid or the presence of microbial biofilms at the tissue surface (Bang et al., 2003, Cooper et al., 2010b).

To date, no clear consensus has been reached on the evolution of the resistance of honey in bacteria. For instance, the authors Blair et al (2009) and Cooper et al (2010) have demonstrated that there is no resistance to Manuka honey with short- and long-term trials against different species, as opposed to findings by Camplin and Maddocks (2014) and Lu et al (2019) that discovered increased resistance in *P. aeruginosa* cells recovered from honey-exposed biofilms *in vitro*. Therefore, how easily bacteria exposed to honey will acquire changes in susceptibility to honey or other antimicrobials remains uncertain. There is some evidence that tolerance to Manuka honey in *P. aeruginosa* affects the susceptibility of rifampicin and imipenem (Camplin and Maddocks, 2014). However, we need to test a broader variety of bacteria and antibiotics on planktonic as well as on sessile growth mode to understand the general picture of how honey adaptation may affects antibiotic susceptibility and describe the genetic mechanisms through which bacteria become resistant.

The aim of this doctoral thesis was to investigate if repeated exposure of various chronic wound bacteria to sub-therapeutic concentrations of Manuka honey wound gel could be selected for strains with reduced sensitivity to honey and antibiotics, and to discover whether changes in phenotype and genotype properties may occur as a result of honey adaptation. Three objectives were created in order to achieve this aim: (i) To investigate the susceptibility of a panel of chronic wound isolates to Manuka honey and antibiotics following repeated passaging with Manuka honey wound gel (P10) and compare them to parent strains (P0). In order to assess if the observed susceptibility changes were transient or stable, the honey-adapted strains were subsequently passaged in honey-free media (X10) and reassessing the susceptibility; (ii) To examine phenotypic changes in honey-exposed, planktonic bacteria through comparison to a respective parent strains regarding growth kinetics, biofilm formation, pathogenicity and exotoxin production ; (iii) To better represent the chronic wound setting through investigation of the effect of Manuka honey passaging in biofilms grown using MBEC assay[TM] in presence

of increasing concentrations of Manuka honey wound gel and identify potential phenotypic and genomic adaptations.

6.2 Variable changes in antimicrobial susceptibility in chronic wound isolates after exposure to Manuka honey

In Chapter 3, the bacterial susceptibility changes to Manuka honey and antibiotics were examined following repeated exposure to sub-therapeutic concentrations of Manuka honey wound gel in planktonic and biofilm growth modes. The data in this chapter showed that the test bacteria varied considerably in their changes in sensitivity to antimicrobials, an observation noted between species and strains. For instance, *P. aeruginosa* WIBG 2.2 exhibited a 7-fold increase in MIC towards ciprofloxacin after wound gel exposure, while no changes were observed in *P. aeruginosa* WIBG 1.3. After ten passages with Manuka honey wound gel, 4/8 test bacteria, including *S. aureus* WIBG 1.6, MRSA*, S. epidermidis* and *P. aeruginosa* WIBG 1.3 exhibited marginal changes (≤ 1-fold) in MIC of honey when compared to parent strains. These changes were stable in the absence of Manuka honey wound gel (X10), except for *S. aureus* WIBG 1.6. Despite these unstable adaptations, Bischofberger et el (2020) discovered a group of genes, such as nemAR and clpP, that affect the Manuka honey adaptation and lead to moderate resistance. These genes play a key role in detoxification of methylglyoxal, which has been shown to be the major contributor to the antibacterial activity of Leptospermum honeys (Adams et al., 2008b, Mavric et al., 2008). It has been argued that this type of temporary adaptation is unlikely to play a major role in the survival of the bacteria over the long term (Russell, 2003). Laboratory studies have suggested that stable resistance to honey is difficult to achieve, reverting to baseline sensitivity profiles in the absence of further challenge (Blair et al., 2009, Cooper et al., 2010b).

Antimicrobial compounds may select for mutations within their target sites, which might be the common targets of other antimicrobial agents (Chuanchuen et al., 2001). Therefore, cross-resistance remains an area of concern regarding misuse of antimicrobials (Wales and Davies, 2015). In the current study, *S. epidermidis* showed transient decreases in susceptibility to both tetracycline and erythromycin after ten passages with Manuka honey wound gel. Interestingly, this increase in MIC toward erythromycin was sufficient to cross a clinical breakpoint outlined by Eucast (https://www.eucast.org/clinical_breakpoints). The clinical significance of these findings is unknown. Phenotypic adaptation mechanisms such as the qacA-mediated efflux system in *S. aureus* and *S. epidermidis* may be attributed to transient changes in bacterial susceptibility to antimicrobial agents by reducing the intracellular concentration of these agents (Hassanzadeh et al., 2017). In contrast to the *S. epidermidis* results, an increase in sensitivity to vancomycin at P10 was observed in all tested strains of staphylococci. An enhanced sensitivity to various antibiotics including tetracycline, imipenem, mupirocin, and rifampicin when combined with Manuka honey was observed previously in MRSA and *P. aeruginosa* (Jenkins and Cooper, 2012).

With regards to sessile communities, both strains of passaged *P. aeruginosa* (P10) exhibited transient increase in MBECs towards gentamicin following culture using an MBEC device. This finding is in agreement with Camplin and Maddocks (2014) which showed reduced sensitivities to both imipenem and rifampicin in *P. aeruginosa* cultures derived from honey exposed biofilm (Camplin and Maddocks, 2014). Further research is warranted with regards to the frequency of this observation in a broader selection of *P. aeruginosa* wound strains, but also the underlying mechanism of action for this observation.

6.3 Phenotypic adaptation in bacteria after Manuka honey exposure

Phenotypic adaptation in passaged isolates is rarely investigated for honey, with studies typically focussing on change in sensitivity to a panel of antibiotics. Investigations with other antimicrobials have suggested that such observations can occur independent of changes in sensitivity. The data in chapter 4 showed that the majority of test bacteria exhibited changes in growth metrics when passaged with honey. As such changes in growth rate were taken into account when determining changes in biofilm formation potential and toxin production. This finding supports previous research which found a positive effect of serial passage on growth rate. Both honey-adapted and control isolates of *E. coli* showed increased in growth rates relative to the ancestral strain in the absence of honey after serial passage (Bischofberger et al., 2020).

During this study it was observed that most of the test bacteria demonstrated significant changes in biofilm forming ability after Manuka honey exposure. A reduction in biofilm formation following Manuka honey wound gel exposure was observed in 3/8 strains including *S. aureus* 1.2, MRSA and *S. pyogenes*. In contrast, a significant increase in biofilm formation was observed in 4/8 strains (*S. epidermidis*, both strains of *P. aeruginosa* and *E. coli*). This could be due to the selection of mutants with altered level of fitness resulting in abnormal growth rates that may influence the formation of the biofilm, or possibly due to mutations affecting processes directly engaged in bacterial adhesion and maturation of the biofilm (Gilbert et al., 1990, Latimer et al., 2012). Recent studies found multiple genes mutated in honey-adapted strains which have a recognised role in biofilm development, such as fimA, fimB, nlpD, icaA, icaD and ompR (Kot et al., 2020, Bischofberger et al., 2020). Since biofilm formation is often an essential determinant of virulence (Naves et al., 2008, Fattahi et al., 2015), this may indicate that the bacteria with altered biofilm formation may also have altered pathogenicity.

In the current study, the relative pathogenicity was determined in all tested bacteria using *Galleria mellonella* waxworms model. After ten passages with Manuka honey, variable effects on pathogenesis, especially between strains of the staphylococci were noted. *S. aureus* WIBG 1.6 exhibited a significant decrease in virulence at P10 which occurred in conjunction with the generation of non-pigmented colonies, reduced haemolytic potential as well as DNase and coagulase activities. These findings are in keeping with previous studies following passage adaptation to triclosan, vancomycin and daptomycin (Latimer et al., 2012, Peleg et al., 2009, Cameron et al., 2015). In contrast, a significant increase in virulence was observed in some of the tested bacteria in this study including *S. aureus* 1.2 (P<0.05), *S. epidermidis* (P<0.01) and *P. aeruginosa* 1.3 (P<0.001) after growth in the presence of honey. After exposure to antimicrobial agents, numerous factors may enhance the bacterial virulence such as increased expression of virulence factors and/or increased biofilm formation. For example, the enhanced virulence of *P. aeruginosa* WIBG 1.3 noted in this study was associated with significant increases in haemolysin, pyocyanin production and biofilm formation following adjustment for changes in growth rate. Additionally, both *S. aureus* 1.2 and *S. epidermidis* exhibited significant increase in haemolytic activity that could be the cause of the enhanced virulence observed in these bacteria.

6.4 Genotypic and phenotypic adaptation in biofilm-derived variants isolated from *S. epidermidis* and *P. aeruginosa* biofilm

Biofilms are functional communities where bacteria show specific phenotypes and genotypes that are profoundly different from planktonic cells (Häußler, 2004). In order to better represent this mode of growth in chronic wounds, genomic and phenotypic changes in passaged biofilms were investigated using a reproducible in vitro system, the MBEC device. The MBEC assay system provides an assay that is easily applicable to investigate various antimicrobials

and multiple organisms in a single assay (Ceri et al., 1999). Without the need for pumps, the MBEC assay system generates 96 equivalent biofilms produced under flow conditions and removes a general source of possible contamination. Additionally, MBEC device contains pegs that allow for numerous replicates and frequent passaging during the assessment of antimicrobials effect against biofilms (Ceri et al., 2001, Coenye and Nelis, 2010). The data in chapter 5 showed that both *P. aeruginosa* and *S. epidermidis* biofilms selected for biofilm-specific variants that established after the first and the second passages, respectively in the presence of honey. Colony variants with reduced diameters, reduced growth rate, slightly increase tolerance to Manuka honey, enhanced biofilm formation and virulence were isolated from both *P. aeruginosa* and *S. epidermidis* biofilms following repeated exposure to Manuka honey. Interestingly, these colony variants also exhibited increased tolerance to ciprofloxacin and gentamicin in *Pseudomonas*, while to vancomycin in *S. epidermidis* when compared to the parent and control strains. The increased tolerance to gentamicin in *Pseudomonas* was also observed with planktonic passaging of the same strain (section 3.3.1.2). Such findings are in agreement with Camplin and Maddocks (2014) findings which showed a slow growing isolates with increased tolerance to Manuka honey, imipenem and rifampicin and increased biofilm forming ability isolated from *P. aeruginosa* biofilm after exposure to sub-lethal concentrations of Manuka honey. Reduced susceptibility to antimicrobials in SCVs is conferred through two known mechanisms. First, it has been shown that a reduced growth rate reduces susceptibility to cell-wall targeting antimicrobials by up to 4 times (McNamara and Proctor, 2000). Secondly, the impairment in bacterial electron transport chains (ETC) reduces the potential of the transmembrane resulting in reduced cationic compound uptake (Gilman and Saunders, 1986). Previous studies demonstrated the development of SCVs after repeated exposure to antimicrobials that have significant impact on bacterial pathogenicity and excretion of some virulence factors such as haemolysin, DNase and coagulase (Bayston et al., 2007, Latimer et

al., 2012, Bazaid et al., 2018). Hence, the implications of honey adaptation on the bacterial phenotypic characterisation and pathogenicity when bacteria grow in sessile form were evaluated in this chapter.

Whole genome sequencing was investigated in all biofilm colony variants of *P. aeruginosa* and *S. epidermidis* following repeated sub-lethal exposure to Manuka honey to provide further insights into the underlying mechanisms of such adaptations. Sequencing of *P. aeruginosa* variants showed genomic mutations in chemotaxis regulator (*cheB* and *mcpB*), virulence and quorum sensing regulator (*hudA* and *lasR*) coding genes, as a response to biofilm growth in the presence of honey. Mutations in the *cheB* gene have been shown to induce hyper-piliation (Whitchurch et al., 2004), inhibit swarming and enhance biofilm formation through stimulating matrix production (Caiazza et al., 2007). Previous studies have also found that *cheB/wspF* mutations resulted in elevated c-di-GMP levels, generating rugose small colony morphotypes in cystic fibrosis infections (Hickman et al., 2005, Starkey et al., 2009). Overexpression of the *hudA* gene caused attenuation in the virulence of *P. aeruginosa* in a Drosophila model, which indicate that *hudA* gene may play a role in the pathogenesis of *Pseudomonas* spp. (Kim et al., 2008). *LasR*, is a quorum sensing gene, that positively regulates the production of virulence factors such as elastase (Hamood et al., 1996), protease (Gambello et al., 1993), pyocyanin (Mavrodi et al., 2001) and extracellular polymeric substance (Sakuragi and Kolter, 2007). Such mutations in multiple genes could explain the various phenotypic biofilm adaptations to Manuka honey that were observed in *P. aeruginosa* in the current study.

In *S. epidermidis,* honey-adapted variants exhibited mutations in carbohydrate, sugar and lipid regulator genes (*cdaR*, *scrK*, *lipA*) and mutation in gene coding for surface-associated fibrinogen binding protein (sdrG). These mutations could play an important role in bacterial adhesion (Hartford et al., 2001, Sellman et al., 2008), cell wall integrity (Rismondo et al., 2016, Mehne et al., 2013b), and bacterial virulence (Hu et al., 2012, Tan et al., 2020). *S. epidermidis*

in this study showed an enhanced virulence, biofilm-formation and increased in MBEC level toward vancomycin after biofilm passaging with Manuka honey, therefore, these genes mutation possibly clarify the underlying mechanisms of such adaptations.

6.5 Conclusion

With the increased prevalence of wound infections especially in the diabetic and ageing populations it is likely that the use of Manuka honey-impregnated wound dressings will continue to rise. Therefore, the long-term effects of Manuka honey exposure, in terms of its impact on bacterial susceptibility, biofilm formation and virulence, as well as its effect on sessile communities should be monitored with regards to health outcomes in the patient. The data presented in this thesis suggest that changes on the bacterial susceptibility and virulence can occur in planktonic and sessile form after prolonged exposure to Manuka honey, although such changes are variable between species and strains. Perhaps it will be clinically useful by having a better idea of the underlying cause of the wound infection before applying honey-impregnated wound dressings. However, the changes in antibiotics susceptibility in our study were very rare and the test bacteria generally remained sensitive to in use concentrations of Manuka honey. As a result of biofilm passaging in presence of Manuka honey, both *P. aeruginosa* WIBG 1.3 and *S. epidermidis* generated colony variants with reduced diameter that exhibited reductions in susceptibility to honey and antibiotics as well as increased biofilm formation potential and virulence.

6.6 Future work

Whilst the present study provided insight into the consequences of long-term exposure to Manuka honey, there are several areas of potential future research. As the antibacterial

properties differ between several type of honey depending on the geographic and floral origins, the potential of bacterial adaptation to other types of honey as well as other Manuka honey brands in a broader panel of pathogens warrants further investigation. As the two strains of Pseudomonas in this study showed changes in gentamicin sensitivity after Manuka honey exposure, it is interesting to assess these changes in other numerous strains of *Pseudomonas*. In addition, assessing the long-term effect of methylglyoxal on wound isolates is another important area to study, as methylglyoxal is known as an active component in Manuka honey. Another important practical implication is to evaluate the effect of Manuka honey on antibiotic resistant bacteria, as these organisms are the major obstacle in wound management.

The data generated from this research was achieved through laboratory based investigations. It is important to note such settings do not entirely represent real life scenarios. Thus, the *in vivo* efficacy of Manuka honey treatment would need to be assessed, both in animal models and clinical trials, in order to link between the laboratory results and the patient. To mimic the wound environment, the researcher could use a validated wound models with artificial wound fluid as the media. Although the *in vitro* methods offer a clear indication of bacterial response, the physiological and immunological changes that occur throughout infection are not considered. Furthermore, wound infection *in vivo* is often associated with a mixed organisms and may require numerous interactions between microorganisms that are not accounted for *in vitro* evaluation.

Biofilms play an essential role in the chronicity of wound infection, hence, the impact of chronic Manuka honey exposure on bacterial biofilm is an important field to investigate. Although in this study, we investigated the effect of Manuka honey on single species biofilms using MBEC^{TM} assay, examining these bacteria using different biofilm models is required to better understand the biofilm-associated adaptation. Moreover, assessing the effect of Manuka

honey on mixed species biofilms using continuous culture model is important to reflect the real

environment of the wound bed.

Appendices

Appendices A. Susceptibility tables of Activon tube (Another brand of Manuka honey)

Methods as referred to in sections 3.2.4 and 3.2.5

Table A1: Antibiotic susceptibility of bacteria before and after passaging with Activon

Bacterium	Antibiotics	MIC (mg/l)			MBC (mg/l)		
		P0	P10	X10	P0	P10	X10
S. aureus WIBG 1.2	Vancomycin	0.976	0.976	0.976	15.6	15.6	15.6
	Ciprofloxacin	0.244	0.488	0.488	0.976	1.95	0.976
	Erythromycin	0.488	0.488	0.488	15.6	15.6	15.6
	Fusidic acid	0.488	0.976	0.976	1.95	3.9	1.95
	Ampicillin	2000	2000	2000	ns	2000	2000
	Tetracycline	0.976	0.244	0.244	7.812	3.9	3.9
S. aureus WIBG 1.6	Vancomycin	0.976	0.976	0.976	7.812	3.9	3.9
	Ciprofloxacin	1.95	1.95	1.95	15.6	15.6	15.6
	Erythromycin	31.25	88.38 (31.25)	31.25	62.5	125	62.5
	Fusidic acid	31.25	15.6	31.25	125	62.5	78.74 (29.46)
	Ampicillin	2000	500	1000	2000	1000	1000
	Tetracycline	0.244	**3.9**	0.488	1.95	7.812	1.95
MRSA	Ciprofloxacin	1.95	3.9	1.95	3.9	7.812	3.9
	Fusidic acid	0.122	0.122	0.122	0.976	0.976	0.976
	Ampicillin	2000	1587 (471.4)	2000	ns	2000	ns
	Vancomycin	0.976	0.976	0.976	3.9	3.9	3.9
S. epidermidis	Ciprofloxacin	0.976	0.488	**0.112**	1.95	0.976	**0.244**
	Fusidic acid	0.244	0.387 (0.406)	0.244	1.95	1.95	1.95
	Vancomycin	1.95	1.95	1.95	15.6	7.812	7.812
	Erythromycin	0.488	1.95	0.488	1.95	7.812	3.9
	Tetracycline	7.812	13.90 (7.812)	7.812	15.6	31.25	15.6
S. pyogenes	Ciprofloxacin	0.488	0.488	0.488	0.976	0.976	0.976
	Erythromycin	0.244	0.690 (0.244)	0.244	1.95	0.976	1.95
	Tetracycline	0.244	0.488	0.488	7.812	7.812	7.812
P. aeruginosa WIBG 1.3	Ciprofloxacin	0.29 (0.12)	0.25 (0.847)	0.244	0.976	0.488	0.488
	Gentamicin	0.976	0.976	1.95	3.9	3.9	3.9
	Meropenem	0.976	0.976	0.976	1.95	1.95	1.95
P. aeruginosa WIBG 2.2	Ciprofloxacin	0.030	**0.488**	0.061	0.244	**1.95**	0.244
	Gentamicin	0.976	0.488	0.976	3.9	3.9	3.9
	Meropenem	0.976	0.976	0.976	1.95	1.95	1.95
E. coli WIBG 2.4	Ciprofloxacin	0.02	0.02	0.02	0.122	0.122	0.244
	Gentamicin	0.976	**7.812**	1.95	3.9	**19.6 (8.08)**	3.9
	Meropenem	0.122	0.122	0.122	0.976	0.976	0.488

The data are expressed as geometric means from biologically duplicated experiments, with each comprising technical triplicates. Bold type indicates a $\geq$ 4-fold change when comparing baseline sensitivities (P0) to P10 and X10 values. Standard deviations are given in the parentheses if the data varied between replicates. Non- susceptible (ns) denotes no sensitivity breakpoint determined as the value was in excess of the antimicrobial concentrations used in the broth dilution.

Table A2: Biofilm eradication concentrations for parent and honey passaged bacteria

Bacterium	Antibiotics	MBEC (mg/l)		
		P0	P10	X10
S. aureus WIBG 1.2	Ciprofloxacin	62.5	62.5	62.5
	Vancomycin	62.5	62.5	62.5
S. aureus WIBG 1.6	Ciprofloxacin	62.5	**314.9**	62.5
			(129)	
	Vancomycin	125	125	125
	Ampicillin	16000	8000	16000
MRSA	Ciprofloxacin	15.6	39.37	22.07
			(16.1)	(8.57)
	Vancomycin	15.6	31.25	15.6
	Ampicillin	ns	ns	ns
S. epidermidis	Ciprofloxacin	7.812	7.812	7.812
	Vancomycin	62.5	62.5	62.5
S. pyogenes	Ciprofloxacin	3.9	1.95	1.95
P. aeruginosa WIBG 1.3	Ciprofloxacin	7.812	11.03	7.812
			(4.26)	
	Gentamicin	125	**629.9**	125
			(258)	
	Meropenem	31.25	11.03	7.812
			(4.26)	
P. aeruginosa WIBG 2.2	Ciprofloxacin	3.9	0.976	1.95
	Gentamicin	15.6	44.19	31.25
			(17.1)	
	Meropenem	2.76	5.51	3.9
		(1.07)	(2.14)	
E. coli WIBG 2.4	Ciprofloxacin	0.976	2.75	0.976
			(1.07)	
	Gentamicin	250	55.68	62.5
			(12.75)	
	Meropenem	0.976	0.976	0.976

The data are expressed as geometric means from biologically duplicated experiments, with each comprising technical replicates. Bold type indicates a $\geq$ 4-fold change when comparing baseline sensitivities (P0) to P10 and X10 values. Standard deviations are given in the parentheses if the data varied between replicates. Non-susceptible (ns) denotes no sensitivity breakpoint determined as the value was in excess of the antimicrobial concentrations used in the broth dilution.

Appendices B. Published manuscript of chapter 3 and 4

This Manuscript is generated from data chapters 3 and 4

Jawahir et al. 2020. Exposure to manuka honey wound gel is associated with changes in bacterial virulence and antimicrobial susceptibility. Frontiers in microbiology, https://doi.org/10.3389/fmicb.2020.02036).

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Exposure to a Manuka Honey Wound Gel Is Associated With Changes in Bacterial Virulence and Antimicrobial Susceptibility

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The use of manuka honey for the topical treatment of wounds has increased worldwide owing to its broad spectrum of activity towards bacteria in both planktonic and biofilm growth modes. Despite this, the potential consequences of bacterial exposure to manuka honey, as may occur during the treatment of chronic wounds, are not fully understood. Here, we describe changes in antimicrobial susceptibility and virulence in a panel of bacteria, including wound isolates, following repeated exposure (ten passages) to sub-inhibitory concentrations of a manuka honey based wound gel. Changes in antibiotic sensitivity above 4-fold were predominantly related to increased vancomycin sensitivity in the staphylococci. Interestingly, *Staphylococcus epidermidis* displayed phenotypic resistance to erythromycin following passaging, with susceptibility profiles returning to baseline in the absence of further honey exposure. Changes in susceptibility to the tested wound gel were moderate ($\leq$ 1-fold) when compared to the respective parent strain. In sessile communities, increased biofilm eradication concentrations over 4-fold occurred in a wound isolate of *Pseudomonas aeruginosa* (WIBG 2.2) as evidenced by a 7-fold reduction in gentamicin sensitivity following passaging. With regards to pathogenesis, 4/8 bacteria exhibited enhanced virulence following honey wound gel exposure. In the pseudomonads and *S. epidermidis*, this occurred in conjunction with increased haemolysis and biofilm formation, whilst *P. aeruginosa* also exhibited increased pyocyanin production. Where virulence attenuation was noted in a passaged wound isolate of *S. aureus* (WIBG 1.6), this was concomitant to delayed coagulation and reduced haemolytic potential. Overall, passaging in the presence of a manuka honey wound gel led to changes in antimicrobial sensitivity and virulence that varied between test bacteria.

Keywords: manuka honey, chronic wounds, MIC, biofilm, antimicrobials

INTRODUCTION

Chronic wounds, such as diabetic foot ulcers, are associated with increased morbidity and mortality worldwide (Majtan et al., 2014). A wound is usually considered chronic if it has failed to heal within 8 weeks and leads to significant tissue loss as a result of disruption during the wound healing stages (McCarty et al., 2012). The impairment of wound healing is caused by a range of factors,

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with bacterial infection frequently cited as a major contributor and aggressive treatment is usually required (Edwards and Harding, 2004; Healy and Freedman, 2006; Lu et al., 2014). A rise in the number of antibiotic-resistant bacteria is a cause for concern in wound management and effective control must be accomplished (Bradshaw, 2011).

Honey has been reported to contain over 200 compounds, including sugars, vitamins, amino acids, minerals, enzymes, flavonoids, antioxidants and phenolic acids (Eteraf-Oskouei and Najafi, 2013; Schneider et al., 2013; Alvarez-Suarez et al., 2014; Stephens et al., 2015). Manuka honey (derived from the *Leptospermum scoparium* tree in New Zealand) is frequently applied in the treatment of bacterial infections (Qamar et al., 2017) and exhibits well documented antibacterial properties as a result of various phenolic compounds (Carter et al., 2016; Johnston et al., 2018) and methylglyoxal, the latter following inhibition of bacterial DNA and protein synthesis (Jervis-Bardy et al., 2011; Kilty et al., 2011; Hayes et al., 2018). At bactericidal concentrations, manuka honey has been reported to cause loss of membrane integrity in both Gram positive and negative bacteria, including *Pseudomonas aeruginosa* (Roberts et al., 2012). At subinhibitory concentrations, manuka honey has been shown to inhibit septa formation in the staphylococci (Henriques et al., 2010; Lu et al., 2013) and down regulation of flagella associated genes in pseudomonads (Roberts et al., 2014). This purported broad spectrum of activity offers some utility in the management of chronic wound infections. The microbiology of chronic wounds is complex and incompletely understood, although studies aiming to profile venous leg ulcers have identified both *Staphylococcus aureus* and *P. aeruginosa* in over 90 and 50% of samples, respectively (Davies et al., 2007; Han et al., 2011). Other taxa have also been reported, including *Enterococcus faecalis*, coagulase negative staphylococci, *Streptococcus* spp., members of the *Enterobacteriaceae* and anerobic rods (Han et al., 2011; Oates et al., 2012).

Concerns have been raised regarding co-selection for antibiotic resistance among bacteria exposed to non-antibiotic antimicrobial agents (McBain and Gilbert, 2001; Buffet-Bataillon et al., 2012; Wales and Davies, 2015). For example, laboratory exposure to some disinfectants has been shown to induce bacterial adaptations that may result in decreased susceptibility to one or more antibiotics (Chuanchuen et al., 2001; Forbes et al., 2014, 2015). Such changes may also occur in conjunction with other phenotypic adaptations that affect biofilm formation potential (Latimer et al., 2012; Henly et al., 2019), bacterial fitness (Forbes et al., 2015) and pathogenicity (Latimer et al., 2012; Bazaid et al., 2018; Henly et al., 2019). The effect of sub-lethal exposure to manuka honey has received relatively little research attention although stepwise training experiments using planktonic cultures of *Escherichia coli, P. aeruginosa, S. aureus* and *S. epidermidis* suggest only transient reductions in sensitivity to honey (Blair et al., 2009; Cooper et al., 2010). The anti-biofilm potential of manuka honey has been described (Maddocks et al., 2012; Lu et al., 2019; Roberts et al., 2019), although elevations in imipenem MIC of up to 4-fold and increased biofilm forming potential have been observed in cultures derived from honey exposed sessile communities (Camplin and Maddocks,

2014). Passaging experiments using other antimicrobials have demonstrated that adaptation can manifest in ways other than through changes in drug sensitivity (Latimer et al., 2012; Lu et al., 2014; Henly et al., 2019). Whilst the general understanding of such effects in response to manuka honey are less clear, a handful of gene expression studies support a view of virulence attenuation (Jenkins et al., 2014; Roberts et al., 2014). It must be noted, however, that manuka honey exhibits a complex mode of action that is capable of acting upon multiple cellular target sites with variable cellular responses to honey reported between different bacterial species (Jenkins and Cooper, 2012; Carter et al., 2016; Hayes et al., 2018; Johnston et al., 2018).

The present study aimed to investigate the consequences of bacterial passage in the presence of a manuka honey wound gel. Changes in biofilm formation, bacterial pathogenicity and exotoxin production were determined in conjunction with antimicrobial susceptibility profiling in planktonic and biofilm growth modes.

MATERIALS AND METHODS

Bacteria

Wild-type clinical wound isolates were previously isolated from diabetic foot wounds as part of a previous study (Oates et al., 2014). These included: *Staphylococcus aureus* WIBG 1.2, *Staphylococcus aureus* WIBG 1.6, *Streptococcus pyogenes* WIBG 2.1, *Pseudomonas aeruginosa* WIBG 1.3, *Pseudomonas aeruginosa* WIBG 2.2 and *Escherichia coli* WIBG 2.4. Methicillin-resistant *Staphylococcus aureus* (MRSA) NCTC 11939 was obtained from the National Collection of Type Cultures (Public Health England, Salisbury, United Kingdom). *Staphylococcus epidermidis* ATCC 14990 was acquired from the American Type Culture Collection (LGC Standards, Teddington, United Kingdom).

Chemicals and Media

All dehydrated bacteriological media were purchased from Oxoid (Basingstoke, United Kingdom) and autoclaved at 121°C (15 psi) for 15 minutes holding time prior to use. Medihoney® antibacterial wound gel™ (Derma Sciences, New Jersey, United States) was prepared as a 75% w/v stock solution in sterile distilled water before use. Medihoney® wound gel™ is formulated by the manufacturer and is stated to comprise Medihoney® (80%) and waxes. Antibiotics were prepared as stock solutions (4000 mg/L) in distilled water for the purposes of sensitivity testing and sterilised through syringe filtration (0.22 μM; Milllipore, Watford, United Kingdom) before use. Clindamycin, erythromycin, fusidic acid, gentamicin, meropenem, tetracycline and vancomycin were obtained from Sigma-Aldrich (Dorset, United Kingdom). Ciprofloxacin was purchased from Alfa Aesar (Heysham, United Kingdom).

Long-Term Exposure of Bacteria to Manuka Honey

Bacteria were repeatedly exposed to the manuka honey wound gel using an agar-based diffusion assay (Perez, 1990). In brief,

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500 µL of 75% w/v wound gel solution was aseptically transferred into a 15 mm well, formed at the centre of a Mueller Hinton agar plate. Each parent strain of microorganism (P0) was distributed radially in triplicate around the central well and incubated at 37°C for 48 h. After incubation, the bacteria that exhibited growth at the innermost part of the radial streak were aseptically re-inoculated onto a fresh exposure plate. This procedure was repeated for a total of ten passages (P10). Since 500 µl of diluted wound gel was deposited in the well, the organisms were exposed to the wound gel on a concentration continuum from 75% (v/v) to effetively zero. The culture for the subsequent passage was sampled at the border between bacterial growth and inhibition and as such, the concentration was in all cases subinhibitory and therefore close to the MIC. To assess any permanent or transient changes in bacterial susceptibility, P10 strains were further subcultured a total of ten times in the absence of antimicrobial to create passage X10.

Determination of Antimicrobial Sensitivitiy

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of bacteria were assessed using a broth microdilution method as described previously (Humphreys et al., 2011). Briefly, overnight bacterial cultures were adjusted to an OD_{600} of 0.8 and further diluted 1 in 100. Adjusted bacterial cultures were transferred to 96 well microtiter plates containing dilutions of wound gel solution that varied by 5% (w/v) intervals. Mueller Hinton broth was used as the growth medium for all sensitivity testing. Where MIC determinations were conducted for antibiotics, 100 µl of respective stock solution was transferred to 100 µl of double-strength Mueller Hinton broth to account for sock solutions being prepared in distilled water. Doubling dilutions were then prepared across the plate ordinate (2000–0 mg/L). The plates were incubated aerobically at 37°C for 18 h. The MIC was defined as the lowest concentration of antimicrobial to inhibit visible microbial growth after overnight incubation. To determine the MBC, aliquots of 5 µL were taken from the wells exhibiting no visible turbidity, spot plated onto the surface of a Mueller Hinton agar plate and further incubated overnight at 37°C. The lowest test concentration of antimicrobial that resulted in the absence of bacterial growth was reported as the MBC. Data are presented as means of biologically duplicated experiments, each comprising technical triplicates. Where doubling dilutions have been used, data are presented as geometric means.

Minimum Biofilm Eradication Concentration (MBEC)

MBECs were determined using the MBEC assay^{TM} plate (Innovotech, Edmonton, Canada) (Ceri et al., 1999). Briefly, overnight bacterial cultures were adjusted to an OD_{600} of 0.8, then, further diluted 1 in 100 using sterile Mueller Hinton broth. 100 µL of adjusted bacterial inoculum was then transferred into each well of the MBEC assay^{TM} plate and

incubated at 37°C for 48 h to support biofilm formation on the transposable pegs. Pegged lids were subsequently transferred to an antimicrobial challenge plate containing doubling dilutions of the applicable antibiotic and incubated for 24 h at 37°C. After incubation, the pegged lid was moved to a recovery plate that contained 200 µL of sterile Mueller Hinton broth and sonicated (50 kHz, 5 min) to detach cells from the transposable lid surface using a SC-52TH Sonicator (Sonicor, New York, United States). Recovery plates were incubated for 24 h at 37°C. The minimum biofilm eradication concentrations (MBECs) were defined as the lowest concentration of antibiotic required to eliminate the biofilm. Data are presented as means of biologically duplicated experiments, each comprising technical triplicates.

Crystal Violet Biofilm Assay

The potential to form biofilms was compared in parent, P10 and X10 bacteria using a crystal violet assay. Overnight bacterial cultures were adjusted to an optical density of 0.8 and then diluted 1:100 in Mueller Hinton broth. Aliquots (150 µl) of diluted bacterial culture were transferred to the wells of a sterile 96-well microtiter plate (Corning Ltd., Weisbaden, Germany) and were incubated aerobically for 48 h at 37°C. After 48 h, the liquid in the wells was removed by inversion of the microtiter plate, and the wells were washed twice using 200 µl of sterile phosphate-buffered saline (PBS). The wells were stained with 250 µl of 1% (w/v) crystal violet solution for 1 min, rinsed twice with PBS and left to air dry at room temperature. To solubilise the attached crystal violet, 300 µl of absolute ethanol was added to each well (10 min) before measuring the absorbance (OD_{600}) using a PowerWave^{TM} XS plate reader (BioTek, Swindon, United Kingdom). Data were presented as biofilm units calculated by dividing the absorbance of the crystal violet bound biofilm by a corresponding planktonic OD_{600} in order to adjust for planktonic mass. All data points were plotted and analysed using GraphPad Prism version 7.0 (GraphPad Software, California, United States) and are presented as means of biologically duplicated experiments, each comprising six technical repeats. Differences between parent and passaged bacteria (P0 vs. P10; P0 vs. X10) were determined using a Mann-Whitney test.

Galleria mellonella Pathogenicity Assay

The methodology was performed as described previously (Latimer et al., 2012). Larvae of Galleria mellonella were purchased from Live Foods Direct (Sheffield, United Kingdom) and stored in the dark for a maximum of 7 d. For each treatment group, 10 larvae were randomly assigned and placed in Petri dishes. Overnight suspensions of P0, P10 and X10 bacteria were centrifuged (13,000 rpm, 10 min) and washed twice using sterile PBS. Then, bacterial suspensions were adjusted using a light spectrophotometer and corresponding CFUs determined through viable counting. Briefly, quantification was performed following 1 in 10 serial dilutions in Mueller Hinton broth. Diutions were plated in triplicate onto Mueller Hinton agar and incubated overnight (18 h; 37°C). The

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corresponding standard bacterial inoculae were as follows: *S. aureus* WIBG1.2 (OD$_{600}$ = 0.1, 1.2 × 10^9 CFU/ml); *S. aureus* WIBG1.6 (OD$_{600}$ = 0.1, 1.3 × 10^9 CFU/ml); MRSA (OD$_{600}$ = 0.1, 1.6 × 10^9 CFU/ml); *S. epidermidis* (OD$_{600}$ = 0.1, 5.8 × 10^8 CFU/ml); *S. pyogenes* (OD$_{600}$ = 0.1, 1.4 × 10^8 CFU/ml); *P. aeruginosa* WIBG1.3 (OD$_{600}$ = 0.1 followed by 1:1000000 dilution, 250 CFU/ml), *P. aeruginosa* WIBG2.2 (OD$_{600}$ = 0.1 followed by 1:1000000 dilution, 64 CFU/ml) and *E. coli* (OD$_{600}$ = 0.1 followed by 1:500000 dilution, 1.9 × 10^4 CFU/ml). These dilutions were determined following in-house testing in order to achieve observable kill rates across the 7 d test period. Each of the larvae were injected with 5µl of adjusted bacterial suspension into the hemocele via the last left proleg using a sterile Hamilton syringe (Sigma, Dorset, United Kingdom). Larvae were incubated in a petri dish at 37°C, and the number of surviving individuals recorded daily for up to 7 d. An untreated group (no injection) and a group injected with sterile PBS were used as controls. All experiments were performed as biological duplicates with each assay comprising 10 worms. Tests were terminated when two or more of the control larvae died. The data were presented as Kaplan-Meier survival curves and intra-strain, pairwise comparisons of datasets (P0 vs. P10; P0 vs. X10) were conducted using the log-rank test in Graph Pad Prism 7 (GraphPad Software, California, United States).

Planktonic Growth Rate

Overnight cultures of all bacterial isolates (P0, P10, and X10) were adjusted to an OD$_{600}$ of 0.8, further diluted 1:100 in Mueller Hinton broth and deposited into 96 well plates. To determine the planktonic growth rate of bacteria, the culture plate was placed into a microplate reader (PowerWave^{TM} XS, BioTek, Swindon, United Kingdom) and the optical density was read every hour for 24 h using Gen5^{TM}1.08 software (BioTek, Swindon, United Kingdom). Growth curve data from eight absorbance readings (biological duplicates each comprising 4 technical replicates) were fitted to a standard form of the logistic equation using the R software package Growthcurver (Sprouffske and Wagner, 2016) to determine metrics relating to intrinsic growth rates (r; h^{-1}), carrying capacity (K) and maximum generation time (t_gen; h^{-1}). Pairwise statistical comparisons of generated datasets were performed between parent and passaged mutants (P0 vs. P10; P0 vs. X10) at $P \leq 0.05$ using a Wilcoxon signed-rank test. Comparisons were performed using SPSS version 22 (IBM analytics, New York, United States).

Haemolysin Assay

Haemolytic activity was measured for all strains that showed a significant change in pathogenicity and exhibited haemolysis when grown in blood agar (Latimer et al., 2012). P0, P10 and X10 passaged bacteria were grown in Mueller Hinton Broth overnight at 37°C. The overnight cultures were diluted 1:100 and incubated at 37°C until an OD$_{600}$ of 0.3 was achieved. Then, whole defibrinated horse blood (5% v/v; Oxoid Ltd., Basingstoke, United Kingdom) was added to the samples and also to sterile broth (negative control).

All assay reactions were incubated in a shaking incubator (100 rpm, 37°C) for 3 h. 1 ml aliquots were then removed and centrifuged at 16,000 g for 4 min (1-14 Microfuge, Sigma-Aldrich, Dorset, United Kingdom). Optical density measurements of the supernatant were determined using a light spectrophotometer (540 nm). In order to control for variability in growth rates, haemolytic activity was adjusted according to viable counts. In order to do this, serial dilutions (1 in 10) were performed, plated onto mannitol salt agar and incubated overnight (18 h; 37°C). Percentage heamolysis was expressed as the change in A_{540} (ΔA_{540})/cfu. Statistical comparisons (P0 vs. P10; P0 vs. X10) were performed in GraphPad Prism version 7 (GraphPad Software, California, United States) using a student's unpaired T-test with Welch's correction. Data are presented as means from biologically replicated experiments ($n = 4$).

Coagulase Assay

Overnight cultures of P0, P10, and X10 *S. aureus* (WIBG 1.2 and 1.6), MRSA and *S. epidermidis* were adjusted to an OD$_{600}$ of 0.4. Aliquots (1 ml) were added to 3 ml of rabbit plasma with EDTA (Bactident coagulase, Merck, Darmstadt, Germany) and incubated at 37°C in a water bath. Tubes were examined for signs of coagulation over 3 h and scored on a five-point scale according to the manufacturer's instructions. Assays were performed as biological triplicates. *S. epidermidis* ATCC 14990 was included a negative assay control.

Pyocyanin Assay

Differences in pyocyanin production between parent, P10 and X10 bacteria were performed using a chloroform extraction approach in order to better understand differences in virulence potential before and after honey exposure. Pyocyanin was determined as described elsewhere (Essar et al., 1990). 10-ml of overnight bacterial culture was grown (37°C, 200 rpm

TABLE 1 | Bacterial sensitivities to a manuka honey wound gel before and after passaging.

Bacterium	MIC (%w/v)			MBC (%w/v)		
	(P0)	(P10)	(X10)	(P0)	(P10)	(X10)
S. aureus WIBG 1.2	15	15	15	30	30	30
S. aureus WIBG 1.6	15	7.5 (2.7)	15	30	15	30
MRSA	11.66 (2.5)	7.5 (2.7)	7.5 (2.7)	30	30	30
S. epidermidis	15	30	30	30	60	60
S. pyogenes	15	15	15	30	30	30
P. aeruginosa WIBG 1.3	60	70	70	>70	>70	>70
P.aeruginosa WIBG 2.2	30	30	30	>70	>70	>70
E.coli	30	30	30	>70	>70	>70

Data are presented as means from biologically duplicated experiments each comprising technical triplicates. Standard deviations are given in parenthesis if the data varied between replciates.

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for 24 h) in PB medium (20 g Bacto peptone, 1.4 g $MgCL_2$, 10 g K_2SO_4) to maximize pyocyanin production. 6 ml of chloroform was added to 10 ml of cell-free supernatant and shaken vigorously until the pyocyanin was extracted into the chloroform layer. The chloroform layer was drawn off and vigorously mixed with 2 ml of 0.2 N HCL to give a pink to deep red solution. The absorbance of this extracted solution was measured at OD_{520nm}. The percentage of pyocyanin production was expressed relative to the parent strain as follows: A_{520} (ΔA_{520})/cfu. Viable counts from corresponding 24 h PB cultures were determined as described for the "*Galleria mellonella* assay." Pyocyanin concentrations were were performed in biological triplicates and the data expressed as means using GraphPad Prism version 7 (GraphPad Software, California, United States). Comparisons between parent and passaged bacteria (P0 vs. P10;

P0 vs. X10) were determined using an unpaired *T*-test with Welch's correction.

RESULTS

Manuka Honey Wound Gel Susceptibility in Passaged Bacteria

The data presented in **Table 1** demonstrates the susceptibilities of all tested bacteria to manuka honey wound gel before and after passaging experiments. MICs varied between bacterial test species and strains, ranging from 7.5% (w/v) to 70% (w/v) following broth microdilution. Overall, 4/8 of bacteria tested exhibited a change in MIC when compared to the parent strain. These changes were marginal ($\leq$ 1-fold vs.

TABLE 2 | Antibiotic susceptibilities of bacteria before and after passaging.

Bacterium	Antibiotics	MIC (mg/l)			MBC (mg/l)		
		P0	P10	X10	P0	P10	X10
S. aureus WIBG 1.2	Vancomycin	0.98	**0.12**	0.24	15.6	3.90	15.6
	Ciprofloxacin	0.24	0.24	0.24	0.98	0.49	0.98
	Erythromycin	0.49	0.98	0.98	15.6	31.25	15.60
	Fusidic acid	0.49	0.49	0.49	1.95	3.90	3.90
	Ampicillin	2000	1000	1000	ns	2000	2000
	Tetracycline	0.98	0.45	0.49	7.81	3.90	3.90
S. aureus WIBG 1.6	Vancomycin	0.98	**0.17 (0.06)**	0.49	0.98	1.95	1.95
	Ciprofloxacin	1.95	0.49	1.95	15.60	3.90	15.60
	Erythromycin	31.25	15.6	15.60	62.50	62.50	62.50
	Fusidic acid	31.25	15.6	31.25	125	62.50	62.50
	Ampicillin	2000	1000	1000	2000	2000	2000
	Tetracycline	0.24	0.24	0.35 (0.133)	1.95	1.95	1.95
MRSA	Ciprofloxacin	1.95	1.95	1.95	3.90	3.90	3.90
	Fusidic acid	0.12	0.12	0.12	0.98	1.95	1.95
	Ampicillin	2000	2000	2000	ns	2000	ns
	Vancomycin	0.98	**0.11**	0.24	3.90	1.95	1.95
S. epidermidis	Ciprofloxacin	0.98	0.49	0.49	1.95	0.98	0.98
	Fusidic acid	0.24	0.24	0.24	1.95	1.95	1.95
	Vancomycin	1.95	**0.29 (0.12)**	0.98	15.6	3.90	7.81
	Erythromycin	0.49	**15.6**	0.49	1.95	**15.60**	3.90
	Tetracycline	7.81	**62.50**	15.60	15.60	62.50	31.25
S. pyogenes	Ciprofloxacin	0.49	0.49	0.49	0.98	0.98	0.98
	Erythromycin	0.24	0.98	0.35 (0.14)	1.95	7.81	1.95
	Tetracycline	0.24	0.98	0.49	7.81	15.60	7.81
P. aeruginosa WIBG 1.3	Ciprofloxacin	0.29 (0.12)	0.24	0.24	0.98	0.49	0.98
	Gentamicin	0.98	3.90	1.95	1.95	7.81	1.95
	Meropenem	0.98	0.98	0.98	1.95	1.95	1.95
P. aeruginosa WIBG 2.2	Ciprofloxacin	0.03	**0.24**	0.03	0.24	0.98	0.24
	Gentamicin	0.98	0.49	0.98	3.90	3.90	3.90
	Meropenem	0.98	0.49	0.49	1.95	0.98	1.95
E. coli WIBG 2.4	Ciprofloxacin	0.02	0.02	0.02	0.12	0.24	0.24
	Gentamicin	0.98	3.90	1.95	3.90	15.60	3.90
	Meropenem	0.12	0.12	0.12	0.24	0.24	0.24

Bold type indicates a $\geq$ 4-fold change when comparing baseline sensitivities (P0) to P10 and X10 values. Data are expressed as geometric means from biologically duplicated experiments, with each comprising technical triplicates. Standard deviations are given in the parentheses if the data varied between replicates. Non-susceptible (ns) denotes no sensitivity breakpoint determined as the value was in excess of the antimicrobial concentrations used in the broth dilution.

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baseline) but sustained in the absence of additional gel passaging (X10), except for *S. aureus* WIBG 1.6 which returned to baseline levels.

Antibiotic Susceptibility in Passaged Bacteria

Table 2 illustrates the susceptibility of wound bacteria to tested antibiotics following passage. Changes in sensitivity to at least one antibiotic were observed in all bacteria. MIC fold changes of $\geq$ 4-fold to baseline were less frequent (5/8 bacteria) and typically associated with increased sensitivity of the staphylococci to vancomycin. Of note, *S. epidermidis* exhibited transient reductions in sensitivity to both erythromycin and tetracycline marked by a *c.* 7-fold and 31-fold increase in MIC, respectively. With regards to sessile communities, 7/8 bacteria exhibited changes in biofilm eradication concentration to at least one antibiotic following the investigation of honey passaged isolates in MBEC devices (**Table 3**). Overall, of the MBECs with observable endpoints in P10 bacteria, 14/19 exhibited no change or less than a 1-fold change to baseline data. Generally, changes in MBEC in excess of 4-fold were infrequent (2/8 bacteria). Both strains of *P. aeruginosa* reported increased MBECs towards gentamicin with strain WIBG 2.2 representative of a *c.* 7-fold reduction in sensitivity. In contrast, passage X10 of *S. aureus* WIBG 1.6 yielded a *c.* 6-fold increase in ampicillin sensitivity when cultured as a biofilm.

Relative Pathogenicity of Passaged Bacteria

A *Galleria mellonella* waxworm model was used to determine relative pathogenicity in all tested bacteria (**Figure 1**). P10 passaged strains exhibited increased virulence ($P \leq 0.05$, log-rank test) in 3/8 bacteria (*S. aureus* WIBG 1.2, *S. epidermidis* and *P. aeruginosa* WIBG 1.3; **Figures 1A,D,G**) when compared to parent strains (P0). These changes in pathogenicity were transient with partial or complete reversion in the absence of continued antimicrobial exposure (X10). A small but significant ($P = 0.049$) increase in larval killing was also observed in *E. coli* (X10) when compared to baseline data (**Figure 1F**). In contrast, a significant attenuation in pathogenicity was observed in the P10 of *S. aureus* WIBG 1.6 (**Figure 1B**).

Impact of Manuka Honey Wound Gel Passaging on Bacterial Biofilm Formation

A crystal violet assay was used to determine biofilm formation for all bacteria before and after repeated honey exposure and following ten passages in a honey-free medium (**Figure 2**). Overall, 3/8 strains exhibited significant reductions in biofilm formation following wound gel passaging (*S. aureus* WIBG 1.2, MRSA and *S. pyogenes*). In contrast, 4/8 strains, including *S. epidermidis*, *P. aeruginosa* (WIBG 1.3, 2.2) and *E. coli* showed a significant increase in biofilm formation according to the crystal violet assay. Reversion to baseline data was observed regarding biofilms formed by X10 *S. epidermidis* and X10 *P. aeruginosa* WIBG 1.3.

TABLE 3 | Biofilm eradication concentrations for parent and passaged bacteria.

Bacterium	Antibiotics	MBEC (mg/l)		
		P0	P10	X10
S. aureus WIBG 1.2	Ciprofloxacin	62.50	31.30	31.30
	Vancomycin	62.50	62.50	62.50
S. aureus WIBG 1.6	Ciprofloxacin	62.50	62.50	62.50
	Vancomycin	125	31.30	62.50
	Ampicillin	16000	4000	**2244 (816)**
MRSA	Ciprofloxacin	15.60	15.60	15.60
	Vancomycin	15.60	62.50	62.50
	Ampicillin	ns	ns	ns
S. epidermidis	Ciprofloxacin	7.81	11.03 (4.26)	7.81
	Vancomycin	62.50	31.25	62.50
S. pyogenes	Ciprofloxacin	3.90	3.90	3.90
P. aeruginosa WIBG 1.3	Ciprofloxacin	7.81	7.81	7.81
	Gentamicin	125	500	125
	Meropenem	31.30	15.60	15.60
P. aeruginosa WIBG 2.2	Ciprofloxacin	3.90	3.90	4.37 (1.59)
	Gentamicin	15.60	**125**	15.60
	Meropenem	2.76 (1.07)	3.90	3.90
E. coli WIBG 2.4	Ciprofloxacin	0.98	0.49	0.98
	Gentamicin	250	125	125
	Meropenem	0.98	0.98	0.69 (0.27)

Bold type indicates a $\geq$ 4-fold change when comparing baseline sensitivities (P0) to P10 and X10 values. Data are expressed as geometric means from biologically duplicated experiments, with each comprising technical triplicates. Standard deviations are given in the parentheses if the data varied between replicates. Non-susceptible (ns) denotes no sensitivity breakpoint determined as the value was in excess of the antimicrobial concentrations used in the broth dilution.

Changes in Haemolytic Potential Following Passage

The ability of planktonic isolates to lyse erythrocytes was investigated in all passaged isolates that demonstrated: (i) a significant change in pathogenicity assay according to log-rank testing; (ii) observable haemolytic activity when incubated on blood supplemented agar and is illustrated in **Figures 3A–C**. In comparison to the parent, *P. aeruginosa* WIBG 1.3 (P10) showed a significant and sustained increase in haemolytic activity following passaging in the presence of manuka honey wound gel. Similar increases in haemolytic potential were also observed for *S. epidermidis,* although such observations were transient and marked by a small but significant reduction in haemolysis in strain X10. In contrast, attenuated haemolysis was noted in *S. aureus* WIBG 1.6 following wound gel passaging equivalent to 50% that of the progenitor strain.

Modelling of Growth Curve Data

Of the eight strains examined for alterations in growth curve metrics, 5/8 showed significant changes in either carrying

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FIGURE 1 | Kaplan Meir curves illustrating percentage survival following injection of *Galleria mellonella* (wax moth) with sterile PBS (solid black line), parent (P0, green dotted line), passaged (P10, purple dotted line), and X10 (orange dotted line) bacteria. Each curve represents a different test bacterium as follows: *S. aureus* WIBG 1.2 **(A)**, *S. aureus* WIBG 1.6 **(B)**, MRSA **(C)**, *S. epidermidis* **(D)**, *S. pyogenes* **(E)**, *E. coli* **(F)**, *P. aeruginosa* WIBG 1.3 **(G)**, *P. aeruginosa* WIBG 2.2 **(H)**. Significant differences in virulence following pairwise comparison with parent strain denoted as *, **, and *** ($P \leq 0.05$, 0.01, and 0.001, respectively).

capacity, intrinsic growth rate or generation time after manuka honey wound gel exposure (**Table 4**). *S. aureus* WIBG 1.6, *S. aureus* WIBG 1.2 and *E. coli* exhibited a significant increase in both carrying capacity and doubling time with a significant decrease in intrinsic growth rate in both P10 and X10 bacteria.

Additionally, *S. epidermidis* showed an increased doubling time concomitant to a reduction in intrinsic growth rate at X10. In contrast, *P. aeruginosa* WIBG 1.3 exhibited a significant and sustained decrease in both carrying capacity and doubling time following passaging.

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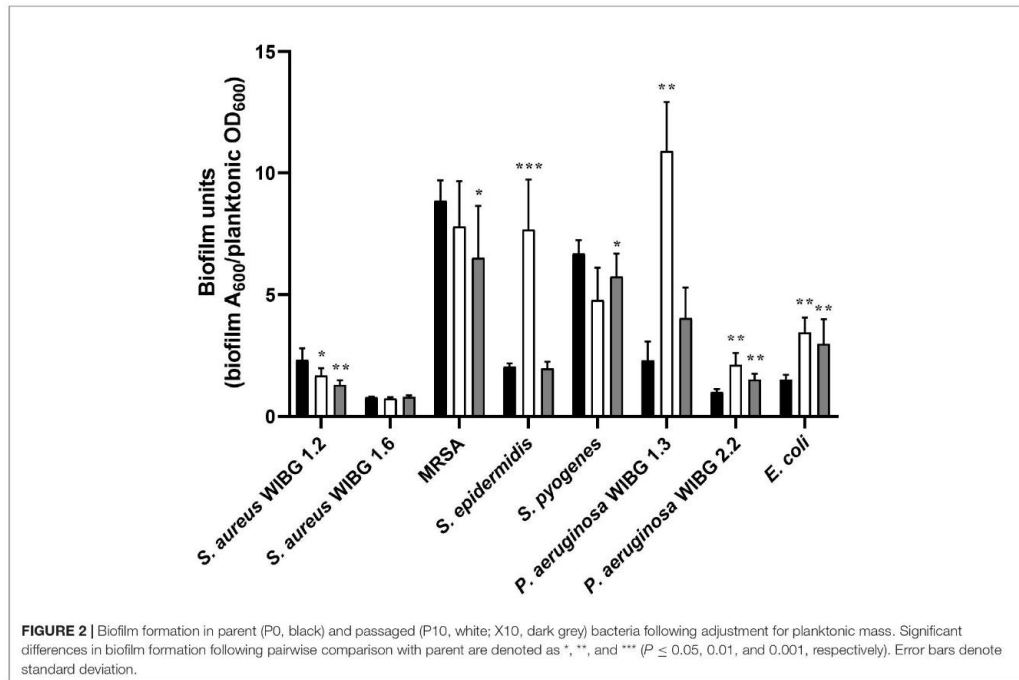
FIGURE 2 | Biofilm formation in parent (P0, black) and passaged (P10, white; X10, dark grey) bacteria following adjustment for planktonic mass. Significant differences in biofilm formation following pairwise comparison with parent are denoted as *, **, and *** ($P \leq 0.05$, 0.01, and 0.001, respectively). Error bars denote standard deviation.

In vitro Coagulase Activity of Pasaged Staphylococci

The levels of coagulase produced by planktonic staphylococci were investigated using a tube coagulase test. After repeated passage with manuka honey wound gel, *S. aureus* WIBG 1.6 showed a delay in coagulation activity in strains P10 and X10, with both showing a positive result after 3 h compared to the parent, which exhibited a positive result after 30 min (**Table 5**). Both WIBG 1.2 and MRSA showed no observable change in coagulation over time. *S. epidermidis* (negative control) exhibited no observable coagulase activity.

Pyocyanin Production in *P. aeruginosa*

P. aeruginosa WIBG 1.3 showed a significant increase in the production of pyocyanin after repeated exposure to manuka honey wound gel (P0 vs. P10, 557.5% $\pm$ 66.3; $P = 0.007$). After removal of the antimicrobial challenge, a reversion in pyocyanin production was observed (P0 vs. X10, 221.5% $\pm$ 106.1; $P = 0.19$). Pyocyanin production was not observed in *P. aeruginosa* 2.2 (**Figure 4**).

DISCUSSION

The exposure of a test panel of bacteria to a wound gel resulted in both increases and decreases in antimicrobial susceptibilities.

Overall, changes were relatively moderate ($\leq$ 7.5% w/v) with regards to sensitivity to the manuka honey wound gel except for a sustained 1-fold increase in MIC in *S. epidermidis*. These observations are in keeping with previous research whereby stepwise resistance training in liquid culture was associated with only minor changes in sensitivity, although observed changes were transient (Cooper et al., 2010). The clinical significance of these observations is unclear with regards to their effect on therapeutic efficacy. The concentrations of manuka honey included in licensed wound care products are typically in excess of the sensitivities reported in the present study (Cooper et al., 2010). Wounds are, however, moist environments which may lead to variable product dilution, as has been discussed by Camplin and Maddocks (Camplin and Maddocks, 2014). Loss of activity following a pH mediated reduction in hydrogen peroxide production may also need to be considered (Bang et al., 2003; Molan and Rhodes, 2015; Cooper, 2016). The low MIC values reported from manuka honey sensitivity studies have been cited in support of the limited effects that wound dilution is likely to impart upon honey efficacy (Molan and Rhodes, 2015). Such effects are, however, less clear regarding the eradication of the biofilm phenotype from a wound environment.

Cross-resistance remains an area of concern regarding overuse of antimicrobials (Wales and Davies, 2015). Adaptation to suboptimal antimicrobial exposure may result in alterations in cell wall permeability or efflux systems that negatively impact

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FIGURE 3 | Haemolytic potential of parent bacterium (P0, black) and manuka honey wound gel passaged (P10, white; X10, dark grey) *S. aureus* WIBG 1.6 **(A)**, *S. epidermidis* **(B)** and *P. aeruginosa* WIBG 1.3 **(C)**. Data are relative to the haemolytic activity of the parent strain. Significant changes in haemolysis following pairwise comparison with baseline data are denoted as * and ** ($P < 0.05$ and 0.01, respectively). Error bars show standard deviation.

upon antibiotic sensitivity profiles. To this end, the exposure of reference strains of *S. aureus* and *P. aeruginosa* to ciprofloxacin, tetracycline and oxacillin have been shown to rapidly generate antibiotic-resistant phenotypes but did not result in observable

cross-resistance to honey (Blair et al., 2009). In the present study, whilst resistance to in-use concentrations of manuka honey were not observed, an additional aim was to investigate the effect of potential honey adaptation on antibiotic susceptibility. Changes in antibiotic profiles were limited, although it must be noted that an increase in MIC occured in *S. epidermidis* to erythromycin. Whilst these observations were transient (i.e.) the phenotype did not persist in the absence of further wound gel passaging, the increase was sufficient to cross a clinical breakpoint so that strain P10 was considered as resistant (The European Committee on Antimicrobial Susceptibility Testing, 2020). Trace levels of macrolides have been detected in honey previously (Bargańska et al., 2011), although, such findings are unlikely to be of relevance in formulations utilised in the healthcare setting given their controlled sourcing and rigorous processing procedures. As such, these data may suggest adaptive resistance via an unknown mechanism.

Changes in antimicrobial sensitivity following honey adaptation in biofilm growth modes have been reported previously (Camplin and Maddocks, 2014). Sessile communities of *P. aeruginosa* were exposed to Medihoney to determine changes in honey inhibitory concentrations, honey biofilm eradication concentrations and antibiotic sensitivities in the residing biofilm biomass. Overall, reduced sensitivities to both imipenem and rifampicin were observed in conjunction with marginal increases in planktonic and sessile honey sensitivities. Biofilms were not directly passaged in the present study but remain of interest as a future research direction. Rather, adapted planktonic cultures were investigated for subsequent changes in MBEC and biofilm-forming potential when tested using a crystal violet assay. Changes in biofilm eradication concentration were marginal in most cases, although a 4-fold reduction in gentamicin sensitivity was observed in a clinical isolate of *P. aeruginosa*. This observation occurred in conjunction with highly significant, but transient, increases in planktonic growth rate and biofilm formation, the later in support of previous investigations (Camplin and Maddocks, 2014).

The use of the *Galleria mellonella* waxworm model in this study enabled an assessment of the virulence potential of bacterial pathogens and suggested variable effects on pathogenesis, particularly between members of the staphylococci. In general, altered virulence in this genus occurred in conjunction with changes in growth metrics, haemolytic activity, coagulation and biofilm formation ability and agrees with studies investigating passaging in the presence of other antimicrobials (Latimer et al., 2012; Bazaid et al., 2018). Interestingly, the enhanced virulence observed in *P. aeruginosa* WIBG 1.3 (P10) is in contrast to previous reports. For example, *P. aeruginosa* wild-type PA14 has been shown to exhibit reduced pyocyanin production following exposure to both raw and heat-treated manuka honey, likely via interaction with the MvfR quorum sensing network (Wang et al., 2012). In the present study, honey adaptation was associated with significant increases in pyocyanin production following chloroform extraction. It must be noted that enhanced virulence may differ between strains as no significant changes in killing were observed in WIBG 2.2, supporting the view that honey is a complex compound comprising active elements capable of

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TABLE 4 | Growth curve metrics for parent and passaged bacteria.

Bacterium	Carrying capacity (k)			Growth rate (h^{-1})			Doubling time (h^{-1})		
	P0	P10	X10	P0	P10	X10	P0	P10	X10
E. coli	0.37 (0.07)	0.35 (0.03)	0.40 (0.04)	0.52 (0.07)	0.35 (0.02)**	0.37 (0.03)**	1.25 (0.16)	1.97 (0.10)**	1.78 (0.36)**
P. aeruginosa WIBG 1.3	0.52 (0.10)	0.30 (0.02)**	0.32 (0.03)**	0.54 (0.21)	1.44 (0.20)**	1.60 (0.36)**	1.43 (0.42)	0.49 (0.07)**	0.45 (0.10)**
P. aeruginosa WIBG2.2	0.44 (0.05)	0.36 (0.09)	0.41 (0.01)	0.77 (0.24)	1.18 (0.58)	0.83 (0.16)	1.06 (0.61)	0.79 (0.28)	0.87 (0.18)
S. aureus WIBG1.2	0.33 (0.08)	0.52 (0.04)**	0.55 (0.03)**	0.48 (0.05)	0.40 (0.02)**	0.40 (0.03)*	1.46 (0.19)	1.75 (0.11)**	1.73 (0.14)*
S. aureus WIBG1.6	0.49 (0.02)	0.60 (0.02)**	0.63 (0.06)**	0.43 (0.02)	0.37 (0.02)**	0.37 (0.03)*	1.60 (0.07)	1.89 (0.12)**	1.89 (0.17)*
MRSA	0.34 (0.03)	0.32 (0.07)	0.42 (0.05)	0.57 (0.15)	0.71 (0.09)	0.51 (0.26)	1.29 (0.31)	0.99 (0.13)	1.30 (0.29)
S. epidermidis	0.22 (0.02)	0.21 (0.01)	0.23 (0.03)	0.53 (0.04)	0.54 (0.06)	0.42 (0.04)**	1.31 (0.10)	1.30 (0.15)	1.66 (0.15)**
S. pyogenes	0.35 (0.02)	0.37 (0.01)	0.44 (0.14)	0.59 (0.13)	0.54 (0.11)	0.48 (0.14)	1.40 (0.42)	1.20 (0.01)	1.61 (1.06)

*Significance denoted as *(P < 0.05) or **(P < 0.01) following pairwise comparison of P10 or X10 to baseline (P0) growth metric data. Standard deviations are given in the parentheses if the data varied between replicates.*

TABLE 5 | Coagulase activity of parent and passaged staphylococci.

Bacteria	Time (h)	Activity for passage		
		P0	P10	X10
S. aureus WIBG 1.2	0.5	++	++	++
	1	+++	+++	+++
	2	++++	++++	++++
	3	++++	++++	++++
S.aureus WIBG 1.6	0.5	++++	-	-
	1	++++	-	+
	2	++++	++	+++
	3	++++	+++ +	+ ++++
MRSA	0.5	++	++	++
	1	+++	+++	+++
	2	++++	++++	++++
	3	++++	++++	++++
S.epidermidis	0.5	-	-	-
	1	-	-	-
	2	-	-	-
	3	-	-	-

Tubes were observed for signs of coagulation over 3 h and scored on a five-point scale according to the manufacturer's guidelines as follows: -, no coagulation detected; +, small separate clots; ++, small combined clots; +++, extensively coagulated clots; ++++, complete coagulation.

FIGURE 4 | Pyocyanin production by *P. aeruginosa* WIBG 1.3 parent (P0, black) and manuka honey wound gel passaged (P10, white; X10, dark grey) bacteria. Data are expressed as a percentage respective to the progenitor (P0) strain. Significant data are represented as ** (P < 0.01). Error bar show standard deviation.

affecting multiple cellular target sites (Jenkins and Cooper, 2012). Whilst previous reports suggest the antimicrobial activity of honey to vary significantly between species, such observations may also be true for phenotypic adaptation between strains and warrants further investigation.

There are some limitations to this study that should be considered. Repeated growth in the absence of honey, analogous to the normal maintenance of bacteria in the laboratory, could conceivably alter bacterial physiology. The method we used has, however, been previously utilised successfully to address a range of research questions (Latimer et al., 2012; Forbes et al., 2014, 2019; Sun et al., 2018) and has been shown not to significantly alter antimicrobial susceptibility or biofilm formation where application without antimicrobial stress has been tested (Henly et al., 2019; Karmakar et al., 2019).

In summary, the repeated laboratory exposure of a test panel of bacteria to manuka honey, in the form of a wound gel, resulted in variable changes in both antimicrobial sensitivity and pathogenesis when compared to a progenitor. This is an important observation as chronic wounds provide an environment where antimicrobial wound dressings may be present *in situ* over prolonged periods of time. However, care must be taken in extrapolating the findings of an *in vitro* study to possible clinical effects. In the current study, phenotypic resistance to erythromycin was observed in a single wound isolate. Cross-resistance following manuka honey exposure has been rarely reported and ongoing work will elucidate the underlying mechanisms. It must be noted though that planktonic bacteria remained susceptible to concentrations of the manuka honey wound gel used in clinical applications, despite repeated exposures. Biofilm formation was also variably affected in passaged bacteria, with notable increases in the pseudomonads and *S. epidermidis*. Whilst this could

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have implications for treatment length, given the propensity for biofilms to form in wounds, significant changes in biofilm sensitivity were generally limited. With the exception of the pseudomonads and gentamicin, a favourable trend of marginal increases in biofilm antibiotic sensitivity were observed in most cases. The underlying mechanisms for such changes are not clear and warrant further investigation. This study also supports the role of additional phenotypic characterisation when investigating adaptation to antimicrobials.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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AUTHOR CONTRIBUTIONS

GH and AM: conceptualisation. JM, GH, and VR: data curation and analysis. JM, GH, AM, VR, and RB: methodology. GH, AM, and RL: supervision. JM, GH, and AM: writing – original draft. GH, JM, AM, and RL: writing – review and editing. All authors contributed to the article and approved the submitted version.

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