Insights into Polymicrobial Communities in Wound Infections

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Abstract

Delayed wound healing, particularly in patients with comorbidities like diabetes, represents a significant health and economic burden, often resulting in a reduced quality of life for these patients. Infection is a key factor in delayed wound healing, with antimicrobial resistance a confounding issue in treatment regimens. Traditional culture-based methods primarily identify predominant pathogens, overlooking the complex wound microbiota, which can influence healing and treatment outcomes. This project explored the dynamics of mixed microbial communities and their antibiotic resistance profiles in both laboratory and clinical settings. An *in vitro* model revealed that mixed bacterial communities can protect susceptible strains from antibiotics, with the extent of this protection varying by antibiotic concentration and bacterial lifestyle (planktonic versus biofilm). The study also optimised host DNA depletion and DNA extraction methods to better analyse the wound microbiome. A 5% saponin-based method significantly reduced human DNA contamination in wound samples ($p<0.0001$), enhancing bacterial detection and increasing the number of unique species identified. This method was then applied in a longitudinal study of the microbiome of diabetic foot ulcers (DFUs). Over five weeks, wound swabs were collected, and the microbiome was analysed using Nanopore sequencing. Key bacteria like *Staphylococcus aureus, Corynebacterium striatum*, and *Finegoldia magna* were identified, alongside a comprehensive microbiome profile and associated antibiotic resistance genes. The study revealed that culture-based diagnostics often miss low-abundance species or overlook certain species, underscoring the need to consider the entire microbial community rather than just commonly culturable pathogens. The findings highlight the importance of advanced molecular techniques in understanding wound microbiota dynamics and resistance profiles. This study challenges the traditional focus on dominant pathogens and highlights the necessity of considering the entire microbial community. Overall, the data presented in this thesis offers insights into the complex microbial communities in wound infections, with implications for future microbiome studies, diagnostics and antibiotic treatment.

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Abbreviations

DFU	Diabetic Foot Ulcer
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
gDNA	genomic Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
PCoA	Principal Coordinate Analysis
ARO	Antibiotic Resistance Ontology
NICE	National Institute for Health and Care Excellence
MIC	Minimum Inhibitory Concentration
NHS	National Health Service
HMP	The Human Microbiome Project
NDFA	National Diabetes Footcare Audit
ECM	Extra-Cellular Matrix
MRSA	Methicillin-Resistant *Staphylococcus aureus*
EPS	Extracellular Polymeric Substance
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight
ESBL	Extended-Spectrum β-lactamases
OD	Optical Density
HHTU	Hull Health Trials Unit
SCV	Small Colony Variant
PBS	Phosphate-Buffered Saline

Conferences and Presentations

Work from this thesis was presented at internal conferences as well as external national and international conferences:

- Poster presentation at the Allam Lecture (13 Sep 2024) – Chapter 5
- Talk at the Centre for Biomedicine Seminar Series (16 Nov 2023) – Chapter 3
- Talk at the Oxford Nanopore Technologies Seminar Day, University of Leeds, UK (27 Sep 2023) – Chapter 4
- Poster presentation at the EMBO | EMBL Symposium - The human microbiome, Germany (20-23 Sep 2023) – Chapter 4
- Talk at MicrobesNG Pop Up Program, Manchester, UK (3 Aug 2023) – Chapter 4
- Talk at the Hull York Medical School PGR Conference (5 Jul 2023) – Chapter 4
- Poster presentation at the Allam Lecture (31 May 2023) – Chapter 4
- Talk at the Centre for Biomedicine Seminar Series (23 Feb 2023) – Chapter 4
- Poster presentation at the Hull York Medical School PGR Conference (14 Jul 2022) – Chapter 5
- Poster presentation at the Research Celebration Event, University of Hull, Hull University Teaching Hospitals and Hull York Medical School (8 Apr 2022) – Chapter 5
- Talk at Hull York Medical School PGR Conference (8-9 Jul 2021) – Project Overview

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Author Declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources. I confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

Chapter 1

Introduction

1.1 Wounds

Wounds and wound infections can lead to significantly reduced quality of life for patients and represent an increasing burden on healthcare systems worldwide. In the UK alone, a study estimated that the National Health Service (NHS) managed 2.2 million wounds, such as diabetic foot ulcers, burn injuries and venous leg ulcers, in the year 2012/2013 (Guest et al., 2015). In 2018, this number was estimated to be 3.8 million (Guest et al., 2020). The National Diabetes Foot Care Audit (NDFA) (2015-2018) in the UK revealed that one in three patients with severe ulcers were admitted to a hospital for a foot-disease-related reason within six months of their first clinical assessment by an expert (NHS England, 2019). In almost 50% of cases, the foot ulcer was still present at 12 weeks after the first assessment by a specialist (NHS England, 2019). In 2018-2023, the NDFA reported >40% severe diabetic ulcers, and 2.6-3.2% of those severe ulcers leading to an amputation within six months of the first clinical assessment by an expert (NHS England, 2023).

Acute wounds show definitive signs of healing in ~4 weeks and progress through the different stages of healing, though they can take up to ~12 weeks to heal completely; a significant proportion fail to heal in this timely and ordered manner and are considered to be chronic wounds (Frykberg & Banks, 2015; Morton, 2016). Previous studies have shown that chronic wounds account for a significant proportion of the total wounds in the UK, and as many as ~30% remain unhealed for long periods, with substantial costs incurred by the NHS in treating wounds (Guest et al., 2015; Guest et al., 2020). Underlying conditions and comorbidities, such as diabetes, obesity, limited/impaired mobility and vascular diseases, can increase the risks of wounds becoming chronic (Beyene et al., 2020; Guo & Dipietro, 2010; Sen, 2019).

A wound represents physical disruption and damage to the skin epithelium and is often accompanied by further damage to the underlying tissue. Wounds can occur due to various reasons like physical trauma, surgery, burns, or diseases such as diabetes. When wounding occurs, the healing process typically follows the four phases of haemostasis, inflammation, proliferation and remodelling (Figure 1.1) (Demidova-Rice et al., 2012). During this process, the damaged skin undergoes numerous changes, and the wound microenvironment promotes healing (Demidova-Rice et al., 2012; Shaw & Martin, 2009; Wilkinson & Hardman, 2020). In the first phase of haemostasis,

a blood clot is formed. A complex immune response is initiated, and the inflammatory phase sees a recruitment of different immune cells to the wound site (Shaw & Martin, 2009; Wilgus et al., 2013). Immune cells release numerous factors and signalling molecules at the wound site, including cytokines, matrix metalloproteinases and growth factors. This promotes migration of fibroblasts and their division, formation of new blood vessels, collagen synthesis and laying down of a new extra-cellular matrix (ECM), re-epithelisation and eventually remodelling of the wound bed (Demidova-Rice et al., 2012; Martin & Nunan, 2015). These steps occur in an overlapping but synchronised manner, leading to wound closure and healing.

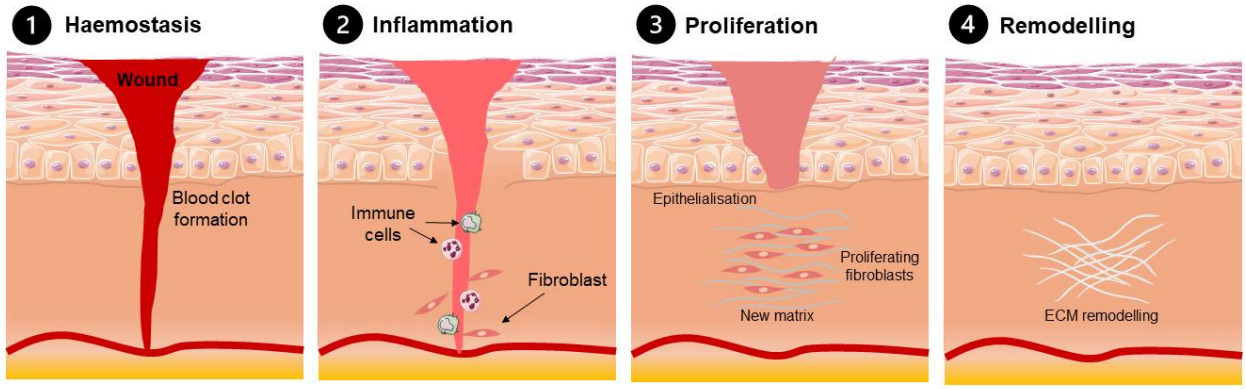

Figure 1.1: The four stages of wound healing *involve a sequential but overlapping process: 1) Haemostasis, where blood clot formation occurs to stop bleeding; 2) Inflammation, characterised by immune cell activity releasing numerous factors and signalling molecules leading to recruitment of fibroblasts; 3) Proliferation, where fibroblasts multiply, collagen synthesis occurs and new tissue forms; and 4) Remodelling, where the tissue strengthens and matures, completing the healing process. (Certain icons and elements in this figure were adapted from Servier Medical Art, licensed under CC BY 4.0)*

The period of wound healing is influenced by external factors such as the origin/cause of the wound, wound depth and size, and also internal factors such as the presence of comorbidities that can include diabetes, renal failure, venous insufficiency, immune disorders amongst others (Beyene et al., 2020). Chronic wounds often display signs of a prolonged inflammatory-proliferative phase, with the presence of cytokines, biochemical factors and immune cells for prolonged periods that prevent healing progression (Barrientos et al., 2008; Guo & Dipietro, 2010; Landén et al., 2016). For

example, prolonged inflammation can lead to an increased number of matrix metalloproteinases in the wound microenvironment that degrade the ECM in the wound bed (Auf Dem Keller & Sabino, 2015; Caley et al., 2015), and this loss of ECM is indeed seen in diabetic foot ulcers (Sutcliffe et al., 2017). This can further prevent cellular migration and wound closure. This complex wound microenvironment, subjected to a prolonged inflammatory phase, is further complicated by the presence of numerous microorganisms.

1.2 The wound microbiome

Wounds are host to diverse microbial communities that include opportunistic wound pathogens and members of the skin commensals. Numerous studies have explored the wound microbiome and identified a plethora of bacteria in different wounds (Giacometti et al., 2000; Johnson et al., 2018; Kalan et al., 2019; Liu et al., 2020; Loesche et al., 2017; Misic et al., 2014; Mohammed et al., 2017; Sloan et al., 2019; Tom et al., 2019; Tomic-Canic et al., 2020; Verbanic et al., 2020; Wolcott et al., 2016). The exact composition of the wound microbiome is diverse across different wound types, origin or cause of the wound, along with other wound characteristics, and influenced by factors such as comorbidities, medication, lifestyle and hygiene. Despite the heterogeneity that exists in wound microbiomes, some key pathogens in wound infections such as *Staphylococcus spp.,* especially *S. aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa* have been identified (Bessa et al., 2015; Dowd et al., 2008b; Fazli et al., 2009; Frank et al., 2009; Giacometti et al., 2000; Gjødsbøl et al., 2006; Kalan et al., 2019; Mohammed et al., 2017; Rahim et al., 2017; Tipton et al., 2017; Tom et al., 2019; Verbanic et al., 2020). *S. aureus* has been most frequently isolated from different wounds, including surgical wounds (Giacometti et al., 2000), burn wounds, and diabetic wounds, amongst others (Gardner et al., 2013; Jneid et al., 2017; Mohammed et al., 2017; Tom et al., 2019). Similarly, *P. aeruginosa* is one of the most commonly found pathogens in wound infections (Kirketerp-Møller et al., 2008; Rahim et al., 2017) and has been isolated from different wound types. Further explorations of the wound microbiome have highlighted the polymicrobial nature as well as diversity that exists in different wound types, with other genera such as *Enterococcus spp., Escherichia coli, Klebsiella spp.,*

Candida spp., Streptococcus spp., Acinetobacter, Corynebacterium and others, being identified (Bowler et al., 2001; Dowd et al., 2008b; Frank et al., 2009; Gardner et al., 2013; Han et al., 2011; Jneid et al., 2017; Kalan et al., 2016; Misic et al., 2014; Price et al., 2009; Sloan et al., 2019; UK Standards for Microbiology Investigations, 2018; Wolcott et al., 2009).

The wound microbiome plays a critical role in both infection and healing (Uberoi et al., 2024), often acting as a double-edged sword in the wound environment. Numerous studies have highlighted that microbial communities in wounds are not merely passive inhabitants but active participants influencing the wound healing process.

Sequencing-based approaches have helped study the wound microbiome in more depth, allowing for the detection of a greater number of genera and more diversity within the microbiome compared to culture-based methods (Han et al., 2011). In a study by Mahnic et al. (2021), cultivation-based detection was found to underestimate bacterial richness in highly diverse communities. Another study also highlighted the importance of molecular methods of bacterial identification in chronic wounds and its improved detection compared to culture-based identification (Rhoads et al., 2012).

The wound microbiome consists of numerous microorganisms, including various pathogens that can lead to wound infections. The wound microbiome plays a crucial role in determining the wound microenvironment, healing state and treatment failure. Various members of the wound microbiome have the potential to cause severe infections in wounds.

1.3 Wound infections

Both acute and chronic wounds are prone to infection, given the presence of microorganisms in all wounds. In normal wound healing, immune cells such as neutrophils play a key role in engulfing and removing pathogens and thus are important in minimising risks of infections (Wilgus et al., 2013). Wounds are colonised with numerous commensals and pathogens. Delayed healing further makes wounds susceptible to infections, as it provides an increased opportunity for pathogens to proliferate, making infections a common occurrence in wounds. Wound infections can

be caused by a range of pathogens commonly found in the wound microbiome, and these infections can affect treatment and healing. Wounds with more than ~10^5 Colony Forming Units (CFU) per gram of tissue are considered to be infected based on some early studies, though this number varies across studies as well as with the infecting agents identified (Bowler et al., 2001; Caldwell, 2020; Tuttle, 2015; Xu et al., 2007).

In the UK, routine treatment for wound infections typically involves the use of antibiotics but may be augmented by the use of antimicrobial dressings and/or ointments. For chronic wounds, recurrent infections are common which can result in multiple antibiotic treatment regimens and extended treatment periods (Howell-Jones et al., 2005). This prescribing pattern can contribute to the development of multidrug resistant organisms present in the wound microbiome. Additionally, wound infections are often characterised by the formation of biofilms (Percival et al., 2015; Wu et al., 2019), which are robust communities of bacteria that are recalcitrant to antibiotics.

First identified as microbial aggregates on teeth (known as ‘plaque’), biofilms are now known to be complex structures of single or multiple species of microorganisms, attached to a surface (Donlan, 2002; Flemming et al., 2016) that are considered ubiquitous in microbial communities. These three-dimensional biofilm structures are broadly composed of extracellular polymeric substance (EPS) matrix in which microbial cells are embedded, and this matrix provides a protective environment. Often these biofilms are polymicrobial, allowing for the sharing of nutrients, horizontal gene transfer and overall mutual benefit to the different species in this community (Gabrilska & Rumbaugh, 2015; Wolcott et al., 2013). Microorganisms in biofilms communicate and co-exist to survive environmental pressures such as immune clearance as well as antibiotics, and are widely known to be associated with infections (James et al., 2008; Malone et al., 2017a; Percival et al., 2018; Percival et al., 2015; Römling & Balsalobre, 2012; Wu et al., 2019; Zhao et al., 2013).

The biofilm matrix, which is comprised of polysaccharides, lipids, extracellular DNA and various proteins, acts as a protective covering for bacteria (Flemming & Wingender, 2010; Karygianni et al., 2020). It not only reduces diffusion of antibiotics into the deeper parts of the biofilm, but also provides a physical barrier to the immune response, thus preventing the killing of the bacteria by immune components and allowing immune evasion (Davies, 2003; Jones & Wozniak, 2017). In addition to this,

bacterial cells within biofilms experience varying microenvironments, with differences in oxygen and nutrient availability, leading to reduced metabolic activity for certain cells in the biofilm (Davies, 2003; de Beer, 1994; Walters et al., 2003). This gives rise to the presence of persisters or dormant bacteria in the biofilm which are tolerant to antibiotics (Keren et al., 2004; Lewis, 2008; Percival, 2011; Wood et al., 2013; Yan & Bassler, 2019). Additionally, the presence of multiple species in a biofilm with different resistance profiles can be advantageous, with horizontal gene transfer of resistance genes, passive resistance to certain antibiotics, metabolic cooperation and enhanced production of virulence factors. All these features combined make biofilms even more recalcitrant to antibiotics compared to planktonic cells, enabling the different species in wounds to withstand antimicrobial challenge. The biofilm state provides enhanced protection to microorganisms from immune clearance and antibiotic treatment, further contributing to the wound microenvironment's inflammation. Thus, biofilms present a challenge in wound management and care and pose a serious threat in wound infections.

As numerous studies have shown, infecting microorganisms exist as biofilms in wounds (Attinger & Wolcott, 2012; James et al., 2008; Malone et al., 2017a; Omar et al., 2017; Zhao et al., 2013). These biofilms are known to be polymicrobial, with different aerobes and anaerobes, Gram-positive and Gram-negative bacteria, and fungal species present (Dowd et al., 2008b; Gabrilska & Rumbaugh, 2015; Percival et al., 2018; Wolcott et al., 2013).

The study of biofilms associated with wound infections using different *in vitro, ex vivo* and *in vivo* models has gained momentum (Brackman & Coenye, 2016; Cardenas-Calderon et al., 2022; Dhekane et al., 2022; Hill et al., 2010; Jensen et al., 2017; Vyas et al., 2022). These models have been helpful in elucidating interspecies interactions in polymicrobial biofilms, identifying virulence factors and demonstrating increased antibiotic tolerance of biofilms. Many of these models focus on the two pathogens most commonly associated with wound infections – *S. aureus* and *P. aeruginosa*. The insights gained from these studies are crucial, as biofilms in wounds can significantly impact the healing outcomes.

Biofilms in wounds can grow and mature, further causing damage to underlying tissue and overwhelming the immune system, prolonging the inflammatory phase and

delaying wound healing (James et al., 2008; Malone et al., 2017a; Omar et al., 2017; Percival et al., 2015; Wu et al., 2019; Zhao et al., 2013). This presence of microorganisms in the form of biofilms, combined with their increased tolerance to antibiotic treatment, can lead to difficulties in treating chronic wound infections.

1.4 Clinical diagnosis of wound infections and antibiotic susceptibilities

Routinely, wounds are inspected by healthcare professionals for suspected infections. Increased inflammation, erythema, pain, purulent discharge and delayed healing are common clinical signs of infections found in wounds (Lipsky et al., 2019; Stevens et al., 2014; The National Institute for Health and Care Excellence (NICE), 2015). While clinical signs may be indicative of infections, further tests are required to identify the putative causative pathogen(s) and their antibiotic susceptibilities.

For this, the healthcare team collects a sample from the patient's wound. There exists some debate about the use of swabs versus biopsies, with certain studies having found that infection assessment does not differ significantly between the two methods of sample collection (Bonham, 2009; Haalboom et al., 2019; Rondas, 2013), and others finding significant differences in the pathogens identified from tissue vs swab samples (Huang et al., 2016; Nelson et al., 2018). For suspected diabetic foot infections, NICE guidelines recommend obtaining a tissue or bone sample from the base of a debrided wound, or a swab sample if the tissue sample cannot be obtained (The National Institute for Health and Care Excellence (NICE), 2015). The most common and widely accepted methods to identify putative pathogens from wound samples are culture-based techniques. This approach typically involves culturing the sample on different types of selective growth media combined with rapid bench testing to allow identification of the causative pathogens.

Once causative pathogen(s) have been identified, their susceptibility to different antibiotics is determined. This is commonly done using disk diffusion assays, and in the UK, the results of these antibiotic testing methods are compared to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint data (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2021; Matuschek et al., 2014).

Another approach has been to use automated testing methods for strain identification and antibiotic susceptibility testing, such as the VITEK system, which has been evaluated for its accuracy and efficiency (Bobenchik et al., 2014; Gupta et al., 2019; Li et al., 2019; Ligozzi et al., 2002; Ling et al., 2001), and many microbiology testing sites use this alongside culture-based identification. The VITEK system uses Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry, a method to measure the mass-to-charge ratio of the ionised sample using the 'time of flight' of the ions. Specific microorganisms generate a signature 'fingerprint', and the characteristic peaks in the spectrum make this fingerprint unique and identifiable for each species (Singhal et al., 2015). MALDI-TOF has been shown to successfully identify pathogens and is typically used for clinical microbiology diagnosis (Hou et al., 2019; Li et al., 2019; Panda et al., 2014; Rodriguez-Lozano et al., 2016; Singhal et al., 2015). However, the utilisation of MALDI-TOF is limited as it requires an axenic culture. Hence, MALD-TOF-based diagnosis in the clinic requires preprocessing steps of bacterial culture for 18-24 hours depending on the bacteria. MALDI-TOF is currently used only for single species identification, though some studies have explored the ability to identify species in mixed cultures. While they have reported some success, this is highly varied and depends on numerous factors such as initial viable cell ratios, number of species, and growth media among others (Florio et al., 2019; Mortelmaier et al., 2019; Zhang et al., 2015). A simulation study using known microbial MALDI-TOF fingerprints to infer the spectrum of a mixed-species culture obtained correct identification for ~61% cases in the simulation (Mahe et al., 2014). The use of MALDI-TOF to identify antibiotic resistance has also been explored and multiple different approaches exist (Axelsson et al., 2020; Flores-Trevino et al., 2019; Florio et al., 2020; Idelevich et al., 2018; Oviano et al., 2014; Vrioni et al., 2018). In these studies, the detection has only been tested for monocultures, and only for one antibiotic at a time. Advances in technology have also led to the emergence of molecular-based detection platforms, such as the Unyvero Platform, which uses multiplex PCR to detect a range of microorganisms and antibiotic resistance markers.

With either automated or traditional culture methods, the growth rate of microorganisms is a limiting factor in the turnaround time. Both methods require plating out of the sample onto culture media followed by an initial 24–48-hour growth. Since this growth is necessary to obtain a pure culture of the pathogen, further tests to

confirm pathogen identity and antibiotic sensitivity testing can be performed only after this initial period, thus increasing the total time before any microbiology test reports can be sent to the clinician.

While culture-based methods remain the gold standard in clinical diagnosis of wound infections, they have limitations beyond the clinical setting. This method restricts identification to only those bacteria that can easily be cultured in the laboratory, focusing on identifying a single causative pathogen, and thus does not take into account the polymicrobial nature of wound infections. Importantly, studies have shown that these methods fail to recapitulate the microbial bioburden and diversity of the wound microbiome (Gardner et al., 2013; Han et al., 2011; Price et al., 2009). With the advancements in sequencing technologies, and the current limitations of culture-based methods, the use of sequencing tools can not only give us more insights into the wound microbiome, but also contribute towards the development of molecular-based diagnostics in clinical microbiology.

1.5 Care, management and antibiotic treatment of wound infections

Wound assessment and understanding of wound aetiology are crucial to inform diagnostic and treatment options, and the T.I.M.E. (tissue, infection/inflammation, moisture, edge of wound) framework is important in performing this assessment (Caroline Dowsett, 2004; Leaper et al., 2012). Practices such as debridement, which involves the removal of dead or infected tissue to promote new tissue growth, and negative pressure wound therapy, which uses suction to remove excess fluid and encourage blood flow to the area, can also play an important role in improving the state of the wound microenvironment. Debridement, combined with surfactant-based solutions, has been shown to impact biofilms in wounds and thus can play a role in infection control (Malone & Swanson, 2017; Wolcott et al., 2010). Effective wound care also emphasises the importance of maintaining moisture balance, preventing infection through proper cleansing and dressing techniques, and ensuring adequate nutritional support for the patient. Infections are a critical concern in wound management, requiring prompt identification and treatment to prevent complications. Antimicrobial

dressings and systemic antibiotics are commonly employed to manage and treat wound infections, aiming to reduce bacterial load and support wound healing.

The general recommended treatment for wound infections is the use of systemic antibiotics, usually given as oral medications (The National Institute for Health and Care Excellence (NICE), 2020). The antibiotic selection primarily depends on antimicrobial sensitivities of the putative causative pathogen identified during microbiology clinical investigations and the severity of the infection alongside local prescribing guidelines and patient drug reactions. In the case of severe infections, antibiotics can be given intravenously (The National Institute for Health and Care Excellence (NICE), 2015; 2020). The first-choice antibiotics are generally broad-spectrum or based upon the most common causative organisms. In wound infections, since the occurrence of *Staphylococcus aureus* and β-lactamase producers is most common, the National Institute of Health and Care Excellence (NICE) guidelines recommend a course of flucloxacillin as the first-choice antibiotic (The National Institute for Health and Care Excellence (NICE), 2015; 2020). Guidelines recommend that samples be taken for microbiology testing, but also state that antibiotic treatment be started as soon as possible in the case of suspected wound infections, leading to a delayed diagnosis, where antibiotics are prescribed first, and reviewed and adjusted based upon the results from microbiology testing results.

In the UK, the overall prescription rate of flucloxacillin has increased by 21% in 10 years (Francis et al., 2016). Significantly higher prescription rates have been associated with patients with chronic wounds; a study in Wales identified that more than 600 prescriptions were issued per 1000 patients with chronic wounds in a year, compared to the non-wound category which received less than 100 prescriptions per 1000 patients in that same period (Howell-Jones et al., 2006). Flucloxacillin (National Center for Biotechnology Information, 2021) is a narrow-spectrum β-lactam antibiotic that is effective against Gram-positive bacteria (Sutherland et al., 1970), but is most commonly used against *Staphylococcus* infections. While most treatment regimens begin with broad-spectrum antibiotics, flucloxacillin is used primarily because *Staphylococcus* species are the most common organisms that cause wound infections. While flucloxacillin is prescribed for *Staphylococcus* infections, MRSA is resistant (Bal, 2005; Sutherland et al., 1970), and hence, when dealing with MRSA

infections, vancomycin or teicoplanin is recommended for leg ulcers and diabetic foot infections (The National Institute for Health and Care Excellence (NICE), 2015; 2020). Like other β-lactams, flucloxacillin inhibits cross-linking between peptides that form peptidoglycan in the bacterial cell wall, thus effectively stopping cell wall synthesis. The side group of the β-lactam ring in flucloxacillin includes an additional isoxazolyl group, which prevents access to the ring and hence degradation by β-lactamases produced by bacteria (Sutherland et al., 1970). With the rise in β-lactamase producing bacteria and their relevance in infections (Brook, 2004; Rice, 2012; Worthington & Melander, 2013), flucloxacillin is highly relevant in wound infection treatment and control. In the case of moderate or more severe diabetic foot infections and severe leg ulcer infections, NICE guidelines recommend the prescription of flucloxacillin at higher doses, or with other antibiotics such as gentamicin or metronidazole, as well as other courses like ceftriaxone with metronidazole (Table 1.1) (The National Institute for Health and Care Excellence (NICE), 2015; 2020). Metronidazole disrupts DNA synthesis in anaerobic bacteria (Edwards, 1993), and thus can help reduce the anaerobic microbial burden in such severe infections. In the case of infections where *P. aeruginosa* is suspected or confirmed, piperacillin, a broad spectrum β-lactam, with tazobactam, a β-lactamase inhibitor, is recommended (The National Institute for Health and Care Excellence (NICE), 2015). Broad-spectrum antibiotics that are recommended work against a range of microorganisms, including Gram-negative bacteria, but they can also lead to selection and further growth of resistant strains in the wound microenvironment. Hence, numerous factors such as the severity of the infection, microbiology testing results, presence of comorbidities such as diabetes, patient age and others should be as taken into consideration when prescribing antimicrobials. If the clinical signs of infection persist despite antibiotic therapy, it is likely the antibiotics are not effective and as such it is important to monitor and re-evaluate the wound regularly to identify any changes seen when antibiotics are taken.

Table 1.1: Key antibiotic recommendations for diabetic foot and leg ulcer infections according to NICE guidelines (The National Institute for Health and Care Excellence (NICE), 2015; 2020)

Type of Wound Infection	Antibiotic Recommended
Mild Diabetic Foot Infection	Flucloxacillin
Moderate to Severe Diabetic Foot Infection	Flucloxacillin with or without Gentamicin and/or Metronidazole
Infected Leg Ulcers	Flucloxacillin (first choice) Co-amoxiclav (second choice)

1.6 Antibiotic resistance

Antibiotics remain the primary treatment option for wound infections. However, antibiotic resistance is a growing global crisis. The emergence of multidrug-resistant and extensively-drug-resistant bacterial strains significantly limits therapeutic options, prolonging infections and increasing morbidity and mortality rates.

In a recently published study that looked at bacteria isolated from 320 wound swab samples and their antibiotic susceptibilities, 91% of *S. aureus* and 73% of *Streptococcus spp.* isolates were resistant to amoxicillin, and all *P. aeruginosa* isolates were resistant to ampicillin, along with a high percentage of *Klebsiella spp.* (73%), and *Enterobacter spp.* (84%) isolates also being resistant to ampicillin (Tom et al., 2019). Methicillin-resistant *S. aureus* (MRSA), commonly associated with wound infections, represents a significant burden with its increased resistance.

Bacteria have developed four distinct mechanisms (the 4 E's) by which they gain resistance to antimicrobials – efflux, enzymes, entry and evasion (Figure 1.2) (Munita

& Arias, 2016; Reygaert, 2018). Efflux mechanisms involve specialised pumps in bacterial cell membranes that actively expel antibiotics, thereby reducing their intracellular concentration and effectiveness. The majority of clinically relevant efflux systems confer multidrug resistance and are categorised into four major groups - resistance nodulation division (RND) family, the major facilitator superfamily (MFS), and the staphylococcal multi-resistance (SMR) and multidrug and toxic compound extrusion (MATE) families (Fernandez & Hancock, 2012; Munita & Arias, 2016; Piddock, 2006).

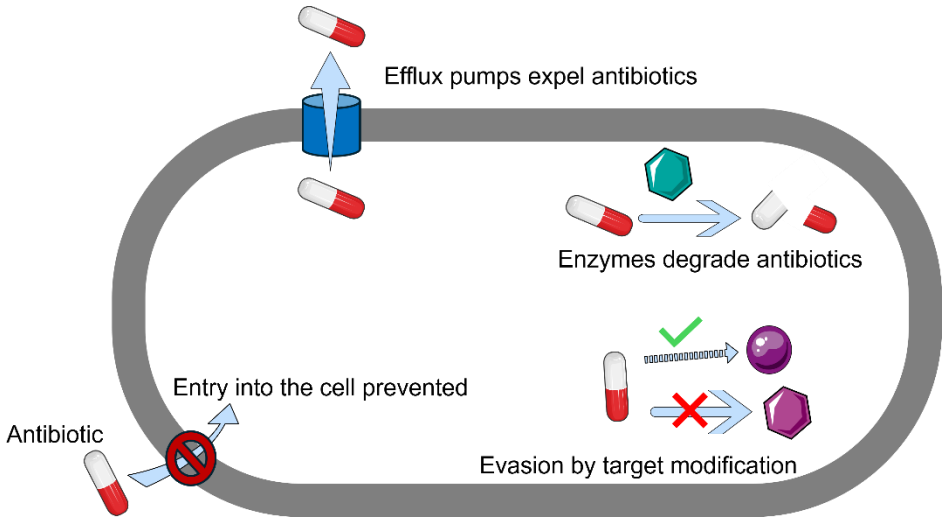

Figure 1.2: The 4E's of antibiotic resistance. *Antibiotic resistance in bacteria can occur through different mechanisms – efflux, enzymes, entry and evasion. (Certain icons and elements in this figure were adapted from Servier Medical Art, licensed under CC BY 4.0)*

Enzymatic resistance occurs when bacteria produce enzymes such as β-lactamases, which hydrolyse antibiotics like penicillins and cephalosporins, rendering them inactive. The β-lactamases hydrolyse the β-lactam ring in this group of antibiotics and thus render the antibiotic ineffective in inhibiting cell wall synthesis. The emergence of strains with β-lactamases and extended-spectrum β-lactamases (ESBLs) (Paterson & Bonomo, 2005; Rawat & Nair, 2010) which confer resistance to 3rd generation cephalosporins and aztreonam, making infection treatment difficult. ESBLs usually derive from genetic mutations and modifications of narrow-spectrum β-lactamases such as TEM-1, TEM-2 and SHV-1.

Entry resistance mechanisms involve alterations in bacterial cell walls or outer membranes, reducing antibiotic permeability and limiting their ability to enter the cell. This can be achieved through modifications in porins or outer membrane proteins that act as barriers against antibiotic entry (Fernandez & Hancock, 2012; Munita & Arias, 2016; Prajapati et al., 2021). Mutations can lead to modifications that result in a structural change in the porin, changes in its expression levels or a loss of a particular porin.

Evasion mechanisms involve changes in bacterial target sites that antibiotics typically bind to, making them less susceptible to drug action. For instance, mutations in bacterial ribosomes can alter the binding site of antibiotics like macrolides, preventing them from inhibiting protein synthesis effectively. An example of this is *mecA*-based resistance in MRSA (Peacock & Paterson, 2015), which is highly relevant for wound infections. The *mecA* gene encodes an altered penicillin-binding protein (PBP2a). This protein has a low affinity for β-lactam antibiotics and thus, the antibiotics are unable to bind to their target (Lim & Strynadka, 2002). Additionally, the *mecA* gene is carried on a mobile genetic element, allowing for its transfer between different bacterial strains.

Antibiotic resistance in wound infections largely determines the effectiveness of antibiotic treatments given to patients. A better understanding of this resistance in the wound microbiome and its influence on healing could change how we think about antibiotic treatment regimens for wound care.

1.7 Resistance profile of the wound microbiome

This increase in antibiotic resistance, combined with the phenomenon of shared resistance, emphasises the need to look at the resistance profile of not only individual pathogens but also the wound microbiome as a whole. While advances in the microbiome field have helped shed light on the wound microbiome, very little is known about the bacterial resistance profile or ‘resistome’ of the wound microbiome and its influence on wound healing. Our current knowledge of antibiotic resistance is limited to the specific pathogens identified in microbiology testing and the use of standard antibiotic susceptibility testing focused on that single pathogen.

When looking at the resistance profile of pathogens associated with wound infections, a majority of studies use disk diffusion assays as the method of resistance identification (Mohammed et al., 2017; Tom et al., 2019). One of the oldest methods of testing antimicrobial susceptibility (Bauer, 1959; Bauer, 1966; Heatley, 1944), this technique is advantageous given the feasibility, relatively low costs and well-standardised methods and is widely used in clinical diagnostics of antibiotic resistance. However, this approach requires isolated colonies to be tested, and like all culture-based methods, can only be used for culturable bacteria. It also fails to capture the resistance profile of the whole microbiome, focusing only on the antibiotic susceptibilities of the causative pathogen isolated from the patient sample. Given the polymicrobial nature of wound infections (Bowler, 2002; Bowler et al., 2001), and interspecies interactions in the wound microenvironment, the presence of other species and their resistance patterns will likely affect the population dynamics of different bacteria in the wound microbiome. The presence of β-lactamase producers can provide 'shared resistance' to non-β-lactamase producing bacterial neighbours (Wang et al., 2023). While there is evidence to suggest that virulent pathogens are linked to delayed healing and treatment failure, the changes that occur in the wound microbiome and its resistance profile over time and in response to antimicrobial treatments have been relatively overlooked.

Hence, studying the microbiome and the resistance profile of the whole microbiome can serve as a crucial first step in understanding resistance in mixed-bacterial communities of the wound that can affect treatment outcomes and healing.

1.8 Nanopore sequencing for wound microbiome characterisation

While previous studies have looked at sequencing-based approaches to explore the microbiome in wounds (Dowd et al., 2008b; Frank et al., 2009; Gardner et al., 2013; Han et al., 2011; Jneid et al., 2017; Price et al., 2009; Wolcott et al., 2009; Wolcott et al., 2016), these have been limited by taxonomic resolution, which can be improved with the use of long-read sequencing techniques. One such method, Nanopore sequencing, employs the use of a nanopore across a membrane to sequence nucleic acids (Jain et al., 2016; Lu et al., 2016). As a single molecule of DNA passes through

the nanopore, the change in electrical conductivity in the pore is measured. These characteristic changes are used to interpret the sequence of the DNA (Deamer et al., 2016). Oxford Nanopore Technologies offers nanopore sequencing through their different commercial products like the MinION sequencer, a palm-sized portable device. Nanopore sequencing has many advantages, including rapid processing of samples with relatively fewer and easier steps, high throughput, ultra-long reads and options to view results in real time. It also has the flexibility of incorporating a PCR amplification if required to target specific regions of the genome or direct sequencing without the need for PCR amplification of the DNA, enabling full genome sequencing. Its long reads and improved sequencing depth allow for better taxonomic resolution when sequencing bacterial DNA (Petrone et al., 2023; Szoboszlay et al., 2023).

Nanopore sequencing has been employed to identify microorganisms in various areas of research but has not been used extensively to explore the wound microbiome. In a study of diabetic heel ulcers, the Oxford Nanopore MinION was used on a subset of samples of DNA extracts from wound swabs (Sloan et al., 2019). Compared to Illumina 16S rRNA sequencing, MinION sequencing gave greater taxonomic resolution and allowed for the identification of antibiotic resistance genes in the sample tested. Though used only on a few samples, this shows that Nanopore sequencing has significant advantages and potential to be explored in understanding the wound microbiome and its resistance profile.

While previous studies have largely identified key pathogenic players in different wound types, this is also influenced by the techniques used to process and analyse the samples. Culture-based methods are limited to the identification of only certain types of bacteria and thus, reduce taxonomic resolution. On the other end of the spectrum, advanced sequencing methods allow better detection of different genera but are greatly influenced by DNA extraction and handling methods. Nanopore sequencing can greatly enhance taxonomic resolution, but various aspects need to be considered in this study before applying this technology to explore the wound microbiome and resistome.

For example, in a study that used pyrosequencing and shotgun Sanger sequencing for ribosomal sequence identification in samples obtained from diabetic wound infections (Dowd et al., 2008a), *Staphylococcus* was the most prevalent species.

However, this study pooled patient samples, and thus, the possibility of bias from a few samples with a higher abundance of *Staphylococcus* could be introduced. Another vital aspect to consider is the presence of host DNA in the sample when sequencing. Irrespective of the type of sample isolated from wound infections, the sample is likely to contain a mix of microbial and host components. Hence, in sequencing-based approaches, a majority of the reads belong to the human genome and do not contribute to the identification of bacterial species. Incorporating a host DNA depletion step in the sample processing can reduce the host reads in the sequencing and allow for greater sequencing depth of the microbial reads. A study that evaluated four commercial microbiome enrichment kits for their ability to deplete host DNA from tissue samples obtained from diabetic foot infections (Heravi et al., 2020) found that the method of host DNA depletion can influence the results obtained from sequencing. While the QIAamp DNA Microbiome kit and Zymo HostZERO microbial DNA kit were highly efficient at depleting host DNA, the NEBNext Microbiome DNA Enrichment kit showed poor efficacy. However, the sample processed with the NEBNext kit was the most similar in its bacterial species profile to a control sample (no host DNA depleted). This indicates that while there is a need to improve sample processing, it is also important to choose techniques that maintain the bacterial species profile as accurately as possible. By applying tools like Nanopore sequencing in the clinical context, our understanding of the wound microbiome and its relation to clinical outcomes can vastly improve.

1.9 Research context and aims

Wound infections pose significant challenges in clinical settings, often leading to prolonged healing times, increased healthcare costs, and heightened patient morbidity. Current diagnostic and treatment strategies for wound infections often overlook the complex interactions within mixed bacterial communities, which can contribute to treatment failure and the emergence of antibiotic-resistant microbes. This research addresses a critical gap in understanding the dynamics of mixed bacterial communities in wound infections, utilising a multifaceted approach (Figure 1.3).

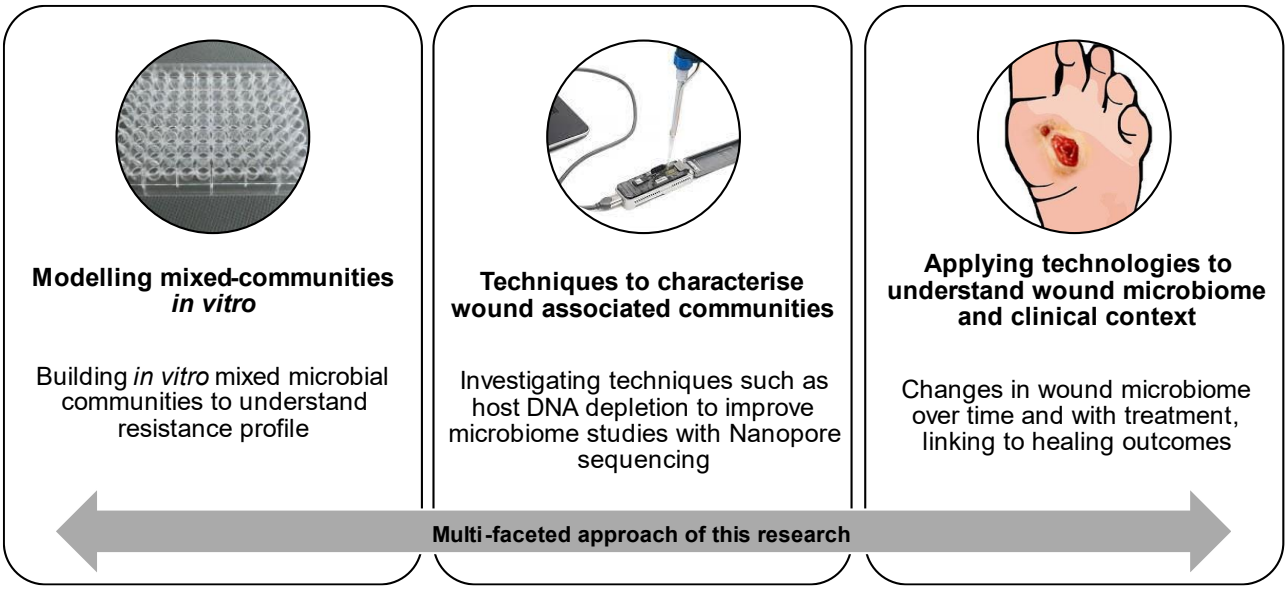

Figure 1.3: Multi-faceted approach used in this research. *This approach was used to shed light on the role of mixed bacterial communities and their resistance profile in wound infections.*

The first approach involves building an *in vitro* mixed-bacterial community model and evaluating the effects of antibiotics on population dynamics. Secondly, a range of host DNA depletion and DNA extraction methods will be assessed for suitability for wound samples. Finally, the optimised host-depletion method will be applied to clinical samples to understand the dynamics of the wound microbiome. The outcomes of this study have the potential to enhance our understanding of mixed-microbial communities and present a novel, in-depth study of the wound microbiome in the context of treatment and healing outcomes.

Chapter 2

General Materials and Methods

2.1 Bacterial strains, culture conditions and storage

All culture techniques were performed under aseptic conditions using a laminar airflow cabinet or a Bunsen burner. All bacterial isolates were archived by freezing (-80°C) as cryo-stocks using the Microbank Bacterial and Fungal Preservation System (Prolab Diagnostics). To revive isolates for testing and generate stock culture plates, a single bead was removed from the cryovial and used to streak onto an appropriate agar plate. Unless stated otherwise, all bacterial strains were cultured on Mueller Hinton agar and broth (ThermoFisher Scientific). Agar plates were incubated for 16-20 hours at 37°C. Stock plates were wrapped in parafilm and stored at 4°C. Sub-cultured stock agar plates were generated from the primary stock culture plate and then further sub-cultured for no more than four passages to minimise phenotypic variations and genetic drift, after which new stock plates were generated from cryo-stocks. Specific bacterial isolates used are stated in respective chapters (Chapter 3 and Chapter 4).

2.2 Optical density and CFU calibration

CFU calibration tests were performed to quantify colony forming units (CFU) relative to the optical density. Briefly, a single colony of each strain was inoculated in separate tubes containing Mueller Hinton broth (for *S. pyogenes* Todd Hewitt broth) and grown overnight (O/N) at 37°C under aerobic conditions. Following incubation, the optical density of the O/N culture/s was measured at 600 nm using a spectrophotometer and adjusted to an optical density of 0.1 OD_{600}. The adjusted cultures were serially diluted and plated (spot plating) onto Mueller Hinton agar or, for *S. pyogenes*, Todd Hewitt agar. The plates were incubated O/N at 37°C, and viable colony counts were performed the following day. Colony counts were calculated using equation (1) formula for each bacterial strain of interest.

Equation 1: $$CFU/ml = \frac{\text{Number of Colonies x Dilution Factor}}{\text{Volume of Culture Plated}}$$

Three biological replicates, with two technical replicates each, were performed. This provided a CFU calibration for each strain, indicating the CFU/mL equivalent of a 0.1 OD culture. These calibrations were used throughout this research to ensure accurate quantification of different strains as needed in Chapters 3 and 4.

2.3 Obtaining CFU counts

Spot plating was used to quantify colony forming units (CFU) throughout this study. Briefly, cultures to be quantified were serially diluted (1 in 10 dilutions using 9 mL sterile media). Each dilution was spot plated using an electronic pipette, releasing five spots of 20 µL each onto the agar and letting the spots dry before incubation at 37°C O/N. The next day, colonies were counted for each spot, and a total colony count was obtained for the 100 µL inoculum placed onto the plate for each serial dilution. Unless otherwise stated, all spot plating was performed with two technical replicates for each biological replicate (three biological replicates). CFU/mL was calculated using the appropriate dilution factors.

2.4 Chemical reagents and plasticware

All chemical reagents used within this study were of certificated analytical grade quality, obtained from Sigma-Aldrich, Poole, UK, unless otherwise stated. Microbial culture media (broth and agar) were provided by Oxoid (ThermoFisher, Basingstoke, UK) and reconstituted using the manufacturer's recommendations. General plasticware was acquired from STARLAB (Milton Keynes, UK) or Fisher Scientific (Loughborough, UK) unless otherwise specified. All molecular assays were carried out using molecular grade water that was DNase-, RNase- and Protease-free (Fisher Scientific, 10490025).

2.5 DNA storage

All extracted DNA was appropriately labelled and stored at -20°C throughout the duration of this study.

2.6 Statistical analysis

All statistical analysis was performed using OriginPro v10.1 or RStudio v4.2.2 (unless otherwise specified). Data was checked for normality and homogeneity of variances as appropriate. If the assumptions were met, a one-way ANOVA followed by post-hoc multiple comparisons with a Tukey test were performed where applicable. In the case of non-parametric data, a Kruskal-Wallis test was performed where appropriate. All p-values less than 0.05 were considered significant. In the visualisation of data, an asterisk (*) was used to indicate significance according to Table 2.1.

Table 2.1: Statistical significance indicated with an asterisk (*)

p-value	Asterisk
<0.05	*
<0.01	**
<0.001	***
<0.0001	****

2.7 Ethics statement

Wound swabs for Chapter 4 were obtained from amputated limbs at Castle Hill Hospital (Hull University Teaching Hospitals NHS Trust) with ethics approval (REC19/NE/0150). The swabs were collected immediately after amputation, when the specimen had been transported to the research laboratory. No patient information was obtained for the purposes of this study.

All wound swabs for Chapter 5 were obtained from patients undergoing consultation and treatment at the Allam Diabetes Centre, Hull Royal Infirmary (Hull University Teaching Hospitals NHS Trust), with research ethics approval (Study Reference Number 21/YH/0272). Participants provided informed consent before sample collection and all patient data was anonymised before sample processing. All patient data was stored securely using the Hull Health Trials Unit (HHTU) Box storage NHS Data Security and Protection Toolkit (Organisation Code - EE133824-HHTU).

Chapter 3

Mixed Microbial Community Population Dynamics Upon Antibiotic Exposure *In Vitro*

3.1 Introduction

3.1.1 Mixed-species communities in wound infections

Wounds are inhabited by a plethora of microorganisms. This microbial colonisation and further growth can lead to serious infections, which in turn affect healing. While clinical diagnosis focuses on identification of causative pathogens, wound infections are polymicrobial in nature (Bessa et al., 2015; Dowd et al., 2008b; Fazli et al., 2009; Frank et al., 2009; Verbanic et al., 2020). In these polymicrobial infections, different microorganisms most often co-exist within the same microenvironment. The interactions between these microorganisms can be cooperative, competitive, or even synergistic, influencing the pathogenicity and severity of the infection.

Various studies have identified the presence of multiple bacteria in wound infections, such as *Staphylococcus, Pseudomonas, Finegoldia and Streptococcus*, along with *Enterococcus spp, Klebsiella pneumoniae, Acinetobacter baumannii,* and *Enterobacter spp*. (ESKAPE pathogens) amongst others (Bowler et al., 2001; Burmølle et al., 2010; Dowd et al., 2008a; Gjødsbøl et al., 2006; James et al., 2008; Malic et al., 2009; Malone et al., 2017a; Puca et al., 2021; Upreti et al., 2018). While most studies focus on bacterial components of the wound community, fungi have also been found within wound infections, with implications for healing and treatment outcomes (Dowd et al., 2013; Lindsay & A., 2018). These microbial communities are most often present in the form of biofilms (Cooper et al., 2014; James et al., 2008; Malone et al., 2017a; Percival et al., 2015). Biofilms represent microbes attached to a surface and protected by an extracellular matrix. Bacteria within biofilms have enhanced protection from antibiotics and host immune clearance and reduced metabolic activity leading to a dormant state (Stewart, 2002). Bacteria within biofilms also have a higher rate of horizontal gene transfer, with a study showing up to 700 times more plasmid uptake in biofilms compared to free-floating counterparts (Krol et al., 2013). This can lead to exchange of resistance genes through these mechanisms and further contribute to treatment failure. Additionally, biofilms are heterogenous in growth rates, have differences in their microenvironment and stress responses and show the presence of persister cells (Bowler et al., 2020; Lewis, 2005; Stewart, 2002). Thus, biofilms can contribute to chronic infections and treatment failure.

The polymicrobial nature of wound infections presents a significant challenge from a clinical perspective (Buch et al., 2019). This heterogeneous nature of this community of microorganisms means that each species could potentially contribute to the overall pathogenicity and, thus, to treatment outcomes. This means that when exposed to antibiotics, the antibiotics act on the whole community of microorganisms and not just the pathogen of concern identified by clinical diagnosis. Thus, treatment outcomes could be influenced by the various microbial species present.

3.1.2 The need for *in vitro* models of mixed-bacterial communities

In this context, the development of accurate laboratory models is crucial to improving our understanding of these polymicrobial infections. Such models provide valuable insights into the population dynamics, interactions, and antibiotic susceptibility profiles of the mixed community, further improving our understanding of the underlying mechanisms driving wound infections and implications for healing. Various wound infection models make use of animals (mice and pigs) to mimic infections and incorporate host factors that will influence healing. Porcine skin, which is a close mimic of human skin, not just because of the structure but also because of similarities in the wound healing process, has been used to develop *in vivo* and *ex vivo* skin and wound infection models (Hirsch et al., 2008; Jensen et al., 2017; Mohiti-Asli et al., 2017; Pastar et al., 2013; Seaton et al., 2015; Summerfield et al., 2015). Studies have shown the attachment of bacteria to the wounded regions of porcine skin (Alves et al., 2018a; Klein et al., 2018; Zurawski et al., 2019), and have used this to test various therapies (Davis et al., 2013; Milho et al., 2019; Williams et al., 2020). *Ex vivo* porcine skin has also been used to develop commercial testing platforms such as the *ex vivo* Burn Wound Model (NAMSA Perfectus Biomed).

While there are advantages, the use of animal models is accompanied by various limitations, including the need for large, expensive, resource-intensive facilities to house, maintain and care for the animals, along with the ethical concerns and further poor transferability of results from animal models to humans. More human-relevant models exist, such as *ex vivo* human skin biopsies (Rakita et al., 2020; Ud-Din & Bayat, 2017; Yoon et al., 2019), commercial models such as HypoSkin (GenoSkin)

and *in vitro* models that incorporate serum, collagen, fibrinogen and other human-relevant factors (Kadam et al., 2021; Price et al., 2016; Slade et al., 2019; Thaarup & Bjarnsholt, 2021; Werthen et al., 2010). However, these models are also associated with higher costs, the need for more resources and ethical approvals. While such human-relevant models are useful, in certain scenarios, there is a need for much simplified models to enable the exploration of preliminary effects and dynamics of key wound infection players.

Further, when exploring interactions in mixed-species microbial communities, there is a need for models offering precise control over conditions, a straightforward testing procedure and cost-efficient methods. These factors facilitate the exploratory nature of such investigations, underscoring the value of simple *in vitro* models over *in vivo* or *ex vivo* counterparts.

3.1.3 *In vitro* models for wound infections

Various studies have used *in vitro* models to explore interspecies interactions and community dynamics in the context of wound infections. Many of these models have focused on *S. aureus* and *P. aeruginosa*, two of the most common wound pathogens (Lichtenberg et al., 2023; Phan et al., 2023; Serra et al., 2015). While these two pathogens are known to co-exist in wound infections, certain studies have shown that *P. aeruginosa* tends to outcompete *S. aureus* in laboratory models (Dalton et al., 2011; Hotterbeekx et al., 2017; Kadam et al., 2021). In planktonic models, these bacteria engage in direct competition for nutrients and space, often exhibiting antagonistic interactions such as the production of inhibitory substances (Hotterbeekx et al., 2017). In biofilm models, the interactions become even more complex. While *P. aeruginosa* can secrete enzymes that disrupt *S. aureus* biofilms, *S. aureus* can persist within these biofilms by exploiting niches, changing metabolic activity and showing increased virulence (Alves et al., 2018b; Hotterbeekx et al., 2017). *S. aureus* also displays the small colony variant (SCV) phenotype, which has reduced metabolic activity (Gounani et al., 2020; Hotterbeekx et al., 2017). Interestingly, this SCV phenotype can enable *S. aureus* to resist antibiotics and evade the host immune response (Guo et al., 2022; Kahl, 2014).

While *P. aeruginosa* and *S. aureus* are important wound pathogens, numerous other bacteria have been identified in wound infections and there is a need to explore the role of these other species in mixed communities further. A study of *S. aureus, P. aeruginosa, S. oralis* and *M. luteus* in mixed-species biofilms found that the different species displayed varying levels of growth, with *S. oralis* showing improved growth compared to its single-species culture, whereas *M. luteus* was not consistently detected, indicating very low levels of growth (Malic et al., 2011). Understanding such interactions is crucial, as they can influence population dynamics within wound communities. Though *E. coli* is frequently identified in wound infections (Puca et al., 2021), very few studies have explored its interactions within polymicrobial communities. Wong (2021) showed that *E. coli* inhibits *S. aureus* in both *in vitro* and *in vivo* models. Another key pathogen that has been studied for its virulence, antibiotic resistance and pathogenesis in the context of wound infections is *A. baumannii* (McConnell et al., 2013; Williams et al., 2020; Zurawski et al., 2019), though its role in mixed-species communities relevant to wounds has not been explored largely. One study showed that planktonic co-culture of *A. baumannii* with *S. aureus* led to a shielding effect against meropenem (Smith et al., 2021), thus highlighting the need to explore population dynamics in mixed-species communities during antibiotic exposure.

While many models study planktonic mixed-species communities, the presence of biofilms in wounds highlights the need to incorporate biofilm states in laboratory models to provide more insights into these communities. Indeed, previous studies have explored mixed-species biofilms, and these models range from simple dual-species co-cultures to more complex communities involving multiple bacterial strains. For example, a four-species biofilm mouse model consisting of *P. aeruginosa, S. aureus, F. magna* and *E. faecalis* showed that while the four species were present in the wound biofilm, there were also clear segregated pockets of attachment for each species in the wound (Dalton et al., 2011). *In vitro* approaches to studying biofilms have also been instrumental in advancing our understanding of biofilm communities. For instance, microtiter plate assays are commonly used for their simplicity and ability to provide quantitative data on biofilm formation and antibiotic susceptibility (Campo-Perez et al., 2023; Haney et al., 2021). The use of static biofilm models, such as the Calgary Biofilm Device (Ceri et al., 1999), enables high-throughput screening of biofilm

responses to various treatments. More complex biofilm models have also incorporated elements other than laboratory media, such as serum, blood cells, and collagen amongst others (Brackman & Coenye, 2016; Kadam et al., 2021; Price et al., 2016; Slade et al., 2019; Sun et al., 2008). For example, Dhekane et al. (2022) used a complex *in vitro* model incorporating human cells (keratinocytes and fibroblasts) and an artificial wound fluid to study the spatial organisation of *S. aureus* and *P. aeruginosa* biofilms, thus incorporating human cell lines with pathogens in wound relevant media. While these complex *in vitro* models can provide novel insights into microbial communities, the use of standard laboratory media remains one of the initial methods of exploring interspecies interactions and population dynamics.

3.1.4 Antibiotic resistance and mixed-species communities

Simple *in vitro* models exploring mixed-species bacterial communities have revealed a complex interplay between different species in the context of antibiotic resistance. For instance, some bacteria within these communities may produce enzymes (β-lactamases) that degrade antibiotics, thus conferring resistance to neighbouring species (Brook, 2009; Liao et al., 2014; Renneberg & Walder, 1989). Moreover, the spatial organisation within mixed-species biofilms as well as differences in metabolic states can impact antibiotic susceptibility (Ibberson et al., 2022; Vega & Gore, 2014). A study of mixed-species biofilms showed that levels *of P. aeruginosa* and *S. aureus* were unaffected in the presence of flucloxacillin and ciprofloxacin at concentrations twice the peak serum concentrations (Hill et al., 2010). In another study, populations containing different strains of *P. aeruginosa* within the same infection showed increased rates of resistance evolution (Diaz Caballero et al., 2023).

A further aspect that exacerbates the risk of emergence of resistant strains is the potential exposure to sub-optimal concentrations of antibiotics. Exposure to these sub-optimal or sub-minimum inhibitory concentration (sub-MIC) levels of antibiotics can have significant implications for the treatment of infections and the development of antibiotic resistance. Sub-MIC concentrations, though not inhibitory, can still exert selective pressures and promote the survival of bacteria with pre-existing resistance mechanisms or facilitate the emergence of novel resistance traits through genetic

mutations or horizontal gene transfer. A study of methicillin-sensitive *S. aureus* strains isolated from wound infections showed that exposure to sub-MIC concentrations resulted in change of resistance phenotypes - strains exposed to sub-MIC concentrations of cefotaxime developed resistance to gentamicin and ciprofloxacin (Bhattacharya et al., 2017). In another study, *Klebsiella pneumoniae* was seen to develop resistance to cephalothin after a 14-day exposure to sub-MIC concentrations of the drug (Anderson et al., 2023). Such sub-MIC exposure to antibiotics can result in the evolution of high levels of resistance. For example, Jorgensen et al. (2013) showed that exposure to sub-MIC levels of ciprofloxacin led to the emergence of strains with an MIC increased to more than 100 times that of the original population.

Sub-MIC exposure can induce bacterial stress responses and lead to alterations in gene expression profiles that may influence virulence or biofilm formation (Bagge et al., 2004; Gutierrez et al., 2013; Nolan & Behrends, 2021), further complicating the wound-healing process. Moreover, prolonged exposure to sub-MIC levels of antibiotics can potentially contribute to the persistence of chronic wound infections by favouring the selection of antibiotic-resistant bacterial subpopulations. Understanding the dynamics of mixed-species bacterial communities *in vitro* can thus provide key insights into antibiotic resistance of these populations.

While various studies have explored certain mixed-species communities, and others have looked at antibiotic resistance in single-species *in vitro* models, it is also crucial to look at antibiotic resistance in the context of mixed-species communities. Previous studies have indeed shown that antibiotic susceptibility is affected by the presence of other members in such mixed-species communities (Adamowicz et al., 2018; Beaudoin et al., 2017; Bottery et al., 2022; Quinn et al., 2022). For example, Dittmer et al. (2023) tested the efficacy of antimicrobial solutions and dressings using a dual-species *in vitro* model consisting of *P. aeruginosa* and *S. aureus* or *P. aeruginosa* and *E. faecium*. In another study, a three-species biofilm (*P. aeruginosa, S. aureus* and *E. faecalis*) on a collagen scaffold was developed and its antibiotic tolerance evaluated (Cardenas-Calderon et al., 2022). Stoffel et al. (2020), using a Lubbock biofilm model and a fungal-bacterial biofilm model, found that the efficacy of various wound gel products was species-dependent. This highlights further the need to explore antimicrobials in the context of multi-species communities.

In summary, the complex and polymicrobial nature of wound infections poses significant challenges for effective treatment. The presence of diverse microbial communities can complicate clinical management of wounds and influence treatment outcomes. The formation of biofilms further exacerbates this issue by providing an environment that enhances bacterial resistance to antibiotics and immune responses. Though treatment regimens focus on clinically identified pathogens, the interactions within these mixed-species communities and their population dynamics can contribute to treatment failures. Understanding these microbial interactions is crucial, as it can shed light on how antibiotics affect the different members of these microbial communities.

3.2 Aims

The aim of this study was to understand population dynamics in mixed-species communities upon antibiotic exposure. The objectives were as follows:

- Develop an *in vitro* two-species model to optimise different growth conditions for planktonic and biofilm states using wound-relevant bacterial strains.
- Expand the two-species model into a three-species model for planktonic and biofilm states and quantify population dynamics.
- Quantify effects on population dynamics in the three-species model when exposed to different antibiotics.

3.3 Materials and Methods

3.3.1 Bacterial isolates and culture conditions

The bacterial isolates used in this chapter are outlined in Table 3.1. AC2, EC3 and MS6 strains were wild-type strains gifted from K. Webster (Microbiology Department, Hull Royal Infirmary). The *S. pyogenes* strain (SP) was a gift from Dr. Cheryl Walter (University of Hull) and was also isolated at the Microbiology department of Hull Royal Infirmary. All bacterial strains were cultured on Mueller Hinton agar and broth (ThermoFisher Scientific), at 37°C unless stated otherwise. *S. pyogenes* was cultured on Todd Hewitt agar and broth (ThermoFisher Scientific), at 37°C unless stated otherwise.

Table 3.1: Bacterial isolates used in this study

Strain
Acinetobacter baumannii (AC2)
Escherichia coli (EC3)
Staphylococcus aureus (MS6)
Streptococcus pyogenes (SP)

3.3.2 Optical density and CFU calibration

The CFU calibrations for optical density described previously (section 2.2) were used to ensure equal inoculums of each strain throughout this chapter.

3.3.3 Minimum Inhibitory Concentration (MIC) assays

To determine the minimum inhibitory concentration (MIC) of penicillin for all strains used in the two-species model, a 96-well plate microdilution MIC assay was performed (Andrews, 2001). Briefly, wells of a 96-well flat-bottomed plate were inoculated with a freshly adjusted culture with an optical density 0.1 OD_{600} in Meuller Hinton broth. Using a szeries of doubling dilutions, a range of concentrations of the antibiotic, ranging from 512 mg/L to 0.0001 mg/L was distributed across the plate. All assay included a positive control (no antibiotic) and negative control (sterile Mueller Hinton broth). The plates were incubated statically for 24 hours at 37°C. Following incubation, plates were then visually examined for growth. The MIC was determined from well with the lowest concentration of antibiotic showing no visible growth and further confirmed by resazurin. Resazurin (0.15 mg/mL) was added to each well in the 96-well plate, and the plate was further incubated at 37°C for 30 minutes. The change in colour of resazurin from blue to pink was used to infer the presence or absence of bacteria and further confirm the MIC. Three biological replicates (with two technical replicates each) were performed.

MIC assays were also performed for the strains of the three-species model (*S. aureus, E. coli* and *S. pyogenes*) as described above. Since the three-species model made use of Mueller Hinton broth supplemented with yeast extract to support the growth of *S. pyogenes*, all MIC assays relevant to the three-species model were also performed using the same supplemented media. A range of concentrations (512 mg/L to 0.0001 mg/L) were tested for the three antibiotics - penicillin, flucloxacillin and doxycycline.

3.3.4 Validation of mixed community growth conditions

3.3.4.1 Two-species mixed community model

A two-species model was used to explore the impact of mixed communities on population dynamics. Two-species mixed communities were tested in two combinations – *A. baumannii* with *S. aureus*, and *E. coli* with *S. aureus*. These initial two-species models served as the primary pilot phase to refine growth conditions and explore the impact of antibiotics on the population survival. First, the effects of mixed-

culture growth were examined in planktonic cultures without any antibiotics. Next, three different substrates were tested for their ability to support biofilm growth. The two-species model was then utilised to evaluate the effects of penicillin on mixed-species biofilm communities. To quantify each strain within the mixed community, viable counts were conducted (procedure outlined in section 2.3) on selective agar for all cultures at respective time points. Mannitol salt agar (ThermoFisher Scientific) was used for the selection of the Gram-positive bacteria (*S. aureus*) from these communities, and MacConkey agar (ThermoFisher Scientific) was used for the selection of Gram-negative bacteria (*A. baumannii* or *E. coli*). The insights gained from this two-species model were subsequently leveraged to develop a three-species model.

3.3.4.1.1 Two-species planktonic culture model

From overnight cultures, each individual strain was diluted to ~10^4 CFU/mL in Mueller Hinton broth. Mixed communities were prepared by combining the diluted cultures of the two species in a 1:1 ratio, with a total culture volume of 10 mL. These communities were statically incubated for 24 hours at 37°C. Single-species cultures were also incubated similarly as controls using 10 mL of the diluted single-species culture. At 24 hours, serial dilutions were performed, and CFU/mL was determined for all cultures using viable counts and respective selective media. The total CFU per planktonic culture was obtained as CFU/mL x 10 mL. The mean of log (total CFU) (±standard deviation) at 24 hours was calculated for each strain in both mixed and mono-species cultures in the two-species model (N=3).

3.3.4.1.2 Two-species biofilm culture model

Three substrates were evaluated for their ability to support biofilm growth using single-species cultures (as outlined in section 3.4.1.2). Briefly, 2 mL of appropriately diluted inoculums of respective bacterial strains was added to a well in a 12-well plate. One substrate was added into each well, ensuring it was submerged in the media. Plates were incubated at 37°C for 24 hours to allow for biofilm growth. After 24 hours, biofilms were dislodged as described in section 3.4.1.2, using glass beads in sterile 1X phosphate-buffered saline (PBS), and serial dilutions were performed, and counts were estimated using spot plating method on selective agar. The optimal substrate was then chosen to evaluate two combinations of mixed-species biofilms as described

for mixed-species planktonic cultures. Biofilm communities were grown on fine celled foam (OASIS®) using Muller Hinton broth, with ~10^5 cells of each species in the combinations under investigation. Similarly, single-species biofilm controls were also grown with ~10^5 cells of the respective species. After 24 hours, biofilms were dislodged from the substrate and viable counts were performed as previously described. All assays were undertaken in biological triplicate with each consisting of two technical replicates. Log (total CFU) per biofilm was calculated as described previously.

3.3.4.1.3 Two-species mixed community biofilm growth in the presence of penicillin

In order to assess the impact of an antibiotic on population dynamics within mixed-species biofilms, biofilm communities were grown on fine celled foam in a 12-well plate as previously described. Penicillin was introduced at the start (0-hour time point) to each well at sub-MIC (0.03 mg/L or 0.04 mg/L) levels based on the MIC of *S. aureus*. Plates were then further incubated statically for 24 hours at 37°C. CFU counts were obtained as described previously at 0 and 24 hours. All assays consisted of three biological replicates consisting of two technical replicates. Viable counts were performed, and log (total CFU) was calculated as previously described. The survival ratio for each strain in mono- and mixed-species was calculated as described in equations (2) and (3) respectively for every antibiotic concentration tested.

Equation 2: $$Survival\ Ratio = \frac{\text{Log (total CFU in mono-species at x } mg/L\ antibiotic)}{\text{Log (total CFU in mono-species with no antibiotic)}}$$

Equation 3: $$Survival\ Ratio = \frac{\text{Log (total CFU in mixed-species at x } mg/L\ antibiotic)}{\text{Log (total CFU in mixed-species with no antibiotic)}}$$

3.3.4.1.4 Modified Hodge Test for detecting β-lactamase production

A Modified Hodge Test was performed according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2009). A 0.5 McFarland dilution of *S. aureus* was prepared and further diluted 1:10. This diluted solution was spread across a Mueller

Hinton agar plate to create a lawn. A sterile disc was placed in the centre of the plate and loaded with 2048 mg/L penicillin. *A. baumannii* and *E. coli* were streaked as straight lines individually from the disc towards the edge of the plate. The plate was incubated at 37°C for 18 hours. After incubation, plates were examined for a cloverleaf-like shape at the intersections of *A. baumannii* or *E. coli* with *S. aureus* within the zone of inhibition.

3.3.4.2 Three-species mixed-culture model

A three-species mixed-culture model was developed using bacterial strains of *E. coli, S. aureus* and *S. pyogenes*. This mixed community was grown in Mueller Hinton broth supplemented with 0.5% Yeast Extract (Fisher Chemicals) (to support the growth of *S. pyogenes*). A planktonic as well as biofilm model was used to test population dynamics over time and in the presence of antibiotic. To quantify each bacterial strain in the mixed community, each mixed culture was serially diluted and plated on selective agars - Mannitol salt agar (Thermo Scientific) for *S. aureus*, MacConkey agar (Thermo Scientific) for *E. coli* and Todd Hewitt Agar (Thermo Scientific) with Streptococcus selective supplement (Oxoid) for *S. pyogenes*.

3.3.4.2.1 Three-species planktonic culture model

The three bacterial strains were grown as single-species cultures and in a mixed community in planktonic culture. Overnight cultures of each strain were diluted to 0.1 OD and further adjusted to ~10^4 CFU/mL. Mixed communities were prepared by mixing the diluted cultures of the two species in a 1:1:1 ratio, with a total culture volume of 10 mL. These communities were grown statically for 24 hours at 37°C. Single-species cultures were also grown similarly as controls using 10 mL of the diluted single-species culture. Serial dilutions were performed after 24 hours, and selective agar was used to quantify counts of each strain. Log (total CFU) per planktonic culture was calculated.

3.3.4.2.2 Three-species biofilm culture model

The three bacterial strains were grown as single-species and in a mixed community in biofilm culture, similar to the two-species biofilm culture model. Briefly, overnight

cultures of each strain were diluted to 0.1 OD and further adjusted to ~10^5 CFU/mL. Mixed communities were prepared by mixing the diluted cultures of the two species in a 1:1:1 ratio, with a total culture volume of 2 mL per well. These communities were grown statically for 24 hours at 37°C. Single-species cultures were also grown similarly as controls using 2 mL of the diluted single-species culture (~10^5 CFU/mL). After 24 hours, biofilms were dislodged as described before, using glass beads in sterile 1X PBS, and serial dilutions were performed, and counts were estimated using spot plating method on selective agar. Log (total CFU) per biofilm was calculated.

3.3.4.2.3 Scanning electron microscopy

To visualise the attachment of bacteria on the fine-celled foam substrate, scanning electron microscopy (SEM) was performed. The biofilm samples were prepared as described above in the three-species biofilm model and incubated at 37°C. Along with the mono-species and mixed-species biofilms, a negative control (containing only Meuller Hinton broth with 0.5% yeast extract) was also included. After 24 hours, the substrates were washed to remove unattached bacteria and culture media from the substrate to prevent crystal/salt formation during SEM sample processing. To wash the substrates, they were lifted from their wells in the 12-well plate and transferred under sterile conditions to a fresh 12-well plate containing autoclaved milliQ water. The substrates were left submerged in water for 10 minutes. Three such washes were performed. Finally, the substrates were transferred to a fresh 12-well plate and submitted to the SEM Facility at the University of Hull for further processing. The SEM Facility prepared the samples for imaging using glutaraldehyde fixation. Briefly, samples were placed in 2.5% glutaraldehyde for two hours, washed with deionised water and then placed in deionised water for 2 hours, removed and rinsed again. Following this, the samples were treated with increasing concentrations of ethanol (from 10% to 100% with increments of 10), for 2-8 hours each. Upon removal from 100% ethanol, the samples were dried using the critical point method.

3.3.4.2.4 Population dynamics in the three-species model in the presence of antibiotics

The three-species model was used to evaluate population dynamics in both planktonic and biofilm cultures in the presence of either penicillin, flucloxacillin or doxycycline. Antibiotics were added to the cultures at 0-hour time point, in varying concentrations

(see Table 3.2). The concentration of antibiotic used was determined by the MICs, with both sub-MIC and above-MIC concentrations being tested. Three-species planktonic and biofilm mixed-cultures (and respective single-species controls) were grown as described above, with the appropriate concentration of antibiotic. After 24 hours of incubation at 37°C, serial dilutions and spot plating on selective agar were performed to determine counts of each strain. Log (total CFU) per planktonic or biofilm culture was calculated. The survival ratio was calculated for each strain at every antibiotic concentration tested as described above (equations (2) and (3)).

Table 3.2: Antibiotics tested in the three-species model

Antibiotic	Concentrations Tested
Penicillin	- 0.008 mg/L - 0.03 mg/L - 0.24 mg/L
Flucloxacillin	- 0.008 mg/L - 0.063 mg/L - 0.5 mg/L
Doxycycline	- 0.03 mg/L - 0.24 mg/L

3.3.5 Statistical analysis

All statistical analysis was performed using OriginPro (v 10.1). Data normality and homogeneity of variances were confirmed using the Anderson-Darling test and the Brown-Forsythe test respectively. A one-way ANOVA followed by a Tukey multiple comparisons test was performed to assess if the means of groups were significantly different.

3.4 Results

3.4.1 Validation of mixed community growth conditions

3.4.1.1 Planktonic growth rates of bacterial strains

To characterise growth rates of the different bacterial strains in this study, growth curve assays were performed. First, the growth rates of the strains chosen for the two-species mixed community were evaluated (*E. coli, S. aureus* and *A. baumannii*). Monitoring of the optical density of planktonic cultures indicated differences in growth rates of the strains. *E. coli* was found to have a much higher growth rate (1.55±0.07/h) than *S. aureus* (0.72±0.07/h) and *A. baumannii* (0.50±0.07/h) (Figure 3.1).

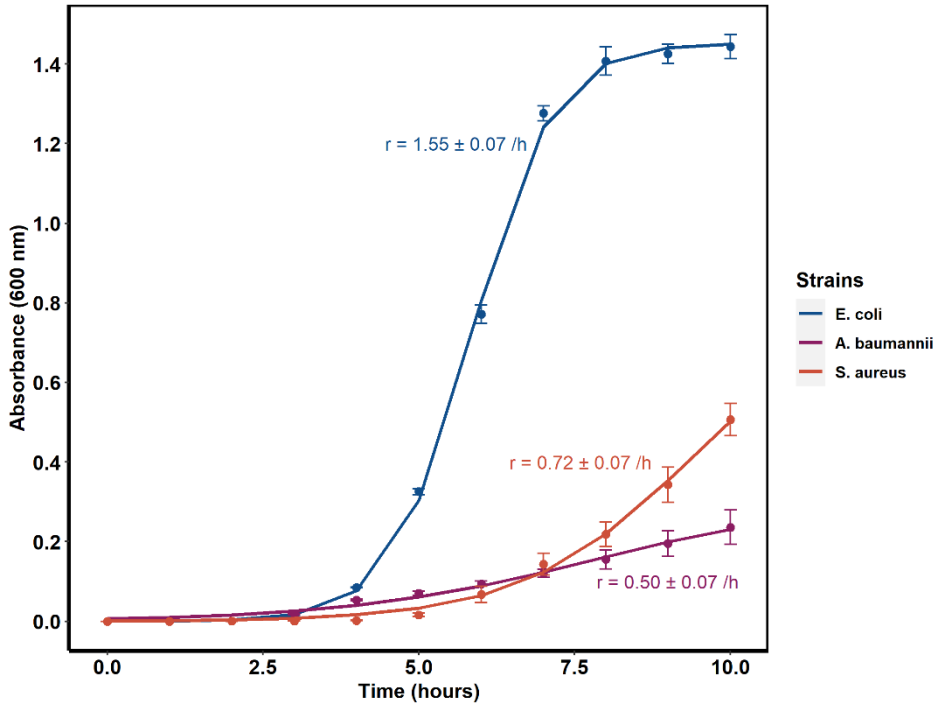

Figure 3.1: Growth curves for three strains used for different in vitro two-species models. *Dots and error bars represent mean±SD values of OD600nm measurements and the lines represent growth prediction from the growthcurver R package, N=3*

In the three-species model, Mueller Hinton broth supplemented with 0.5% yeast extract was used as the base media for all tests. Hence, the growth rates of the three strains were also determined through growth curve analysis in the base media. *E. coli*

exhibited a higher growth rate (1.72±0.08/h) than *S. pyogenes* (0.52±0.07/h) and *S. aureus* (0.79±0.04/h) (Figure 3.2).

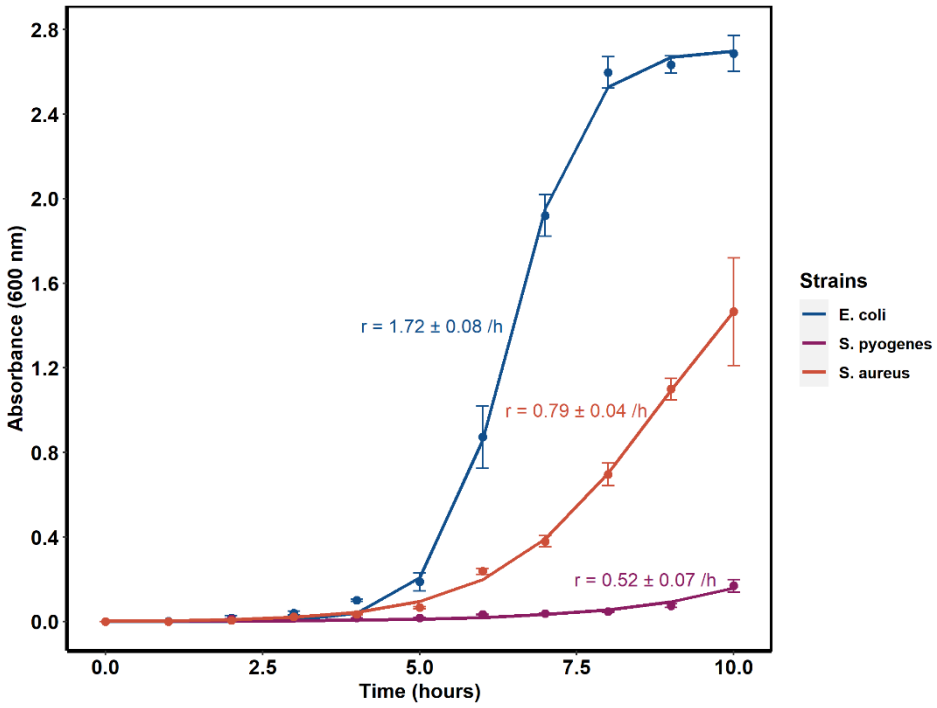

Figure 3.2: Growth curves for three strains used in the three-species models. *Dots and error bars represent mean±SD values of OD600nm measurements and the lines represent growth prediction from the growthcurver R package, N=3*

3.4.1.2 Evaluating substrates for their suitability to develop a biofilm model

To develop a biofilm-relevant model, three substrates were evaluated for their ability to support biofilm growth: a phenolic fine celled foam, a cellulose filter and a monoacetate filter. Each substrate was evaluated for its ability to retain the liquid culture, ease of use and stability after autoclave sterilisation. All three substrates retained their shape and structure after being sterilised in an autoclave. Prior to testing bacterial growth, each substrate underwent immersion in a 12-well plate containing 2 mL of Mueller Hinton broth per plate, allowing for the observation of their respective reactions to liquid submersion. Upon immersion in liquid, the cellulose filter did not retain its former shape and hence was discarded from further analyses.

Biofilms were allowed to grow for 24 hours on the remaining two substrates as described previously. To quantify bacterial growth, biofilms were dislodged by segmenting the substrate into smaller units and subjecting it to further fragmentation

through vortexing with glass beads in PBS. The resulting dislodged biofilm suspension underwent serial dilution and spot plating. Both substrates demonstrated the capability to support growth of *E. coli*, *S. aureus* and *A. baumannii* (Figure 3.3). However, the monoacetate filter was difficult to segment and thus made handling the substrate more challenging during the biofilm dislodgment phase. Hence, based on feasibility of handling, ease of liquid absorption and bacterial growth, the fine-celled foam was chosen as the substrate of choice for an *in vitro* biofilm model.

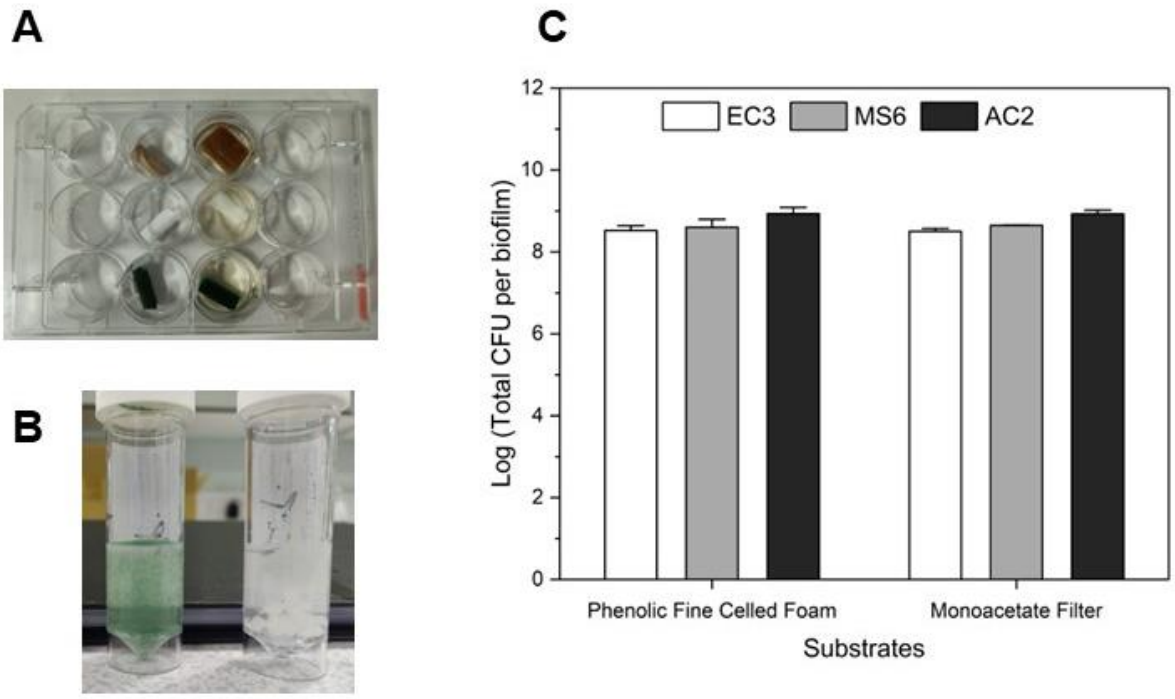

Figure 3.3: Testing different substrates for biofilm growth. *(A) Three substrates were initially evaluated -phenolic fine celled foam, cellulose filter and monoacetate filter. After submerging the substrates in media, the cellulose filter (top wells) was unable to retain its shape and structure and hence was discarded from further analyses. (B) Phenolic foam and filter tip substrates were inoculated with either EC3, MS6 or AC2 and allowed to grow for 24 hours. After 24 hours, the biofilms were dislodge as described resulting a suspension as shown here. The phenolic foam (left) was easily segmented, and fine particles were obtained whereas the monoacetate filter (right) did not segment easily and larger pieces of the substrate remained in the suspension. (C) Log value of the total CFU per biofilm was obtained from serial dilutions and spot plating on each strain, N=2*

3.4.1.3 Minimum Inhibitory Concentration (MIC) assay to determine appropriate antibiotic concentrations for mixed-species community exposure

In order to determine the appropriate antibiotic concentrations at which mixed-species communities would be grown, it was crucial to determine the minimum inhibitory concentration (MIC) of different antibiotics and strains in this study.

For the two-species model, penicillin was the antibiotic of choice. The MIC of penicillin for *S. aureus* was found to be 0.0625 mg/L, whereas *A. baumannii* and *E. coli* showed growth up to 512 mg/L with no visible inhibition (Table 3.3). According to EUCAST guidelines and breakpoints (v14), *Acinetobacter spp. a*nd *Enterobacterales* are resistant to penicillins. Previous characterisation carried out using the VITEK system (unpublished) indicated that these two strains (AC2 and EC3) were resistant to penicillins such as ticarcillin, ticarcillin-clavulanic acid and piperacillin-tazobactam.

Table 3.3: MIC of penicillin for strains used in the two-species model

Strain	MIC
Acinetobacter baumannii	>512 mg/L
Escherichia coli	>512 mg/L
Staphylococcus aureus	0.0625 mg/L

MIC values represent the consistent result obtained across three biological replicates (N=3), each performed with two technical replicates, with no variation observed between experiments.

For the three-species model, MIC assays were performed for *S. aureus*, *E. coli* and *S. pyogenes* for three antibiotics of choice – penicillin, flucloxacillin and doxycycline (Table 3.4).

Table 3.4: MIC of three antibiotics for the three-species model

Strain	Antibiotic	MIC
Staphylococcus aureus	Penicillin	0.063 mg/L
	Flucloxacillin	0.125 mg/L
	Doxycycline	0.063 mg/L
Escherichia coli	Penicillin	>512 mg/L
	Flucloxacillin	>512 mg/L
	Doxycycline	16 mg/L
Streptococcus pyogenes	Penicillin	0.015 mg/L
	Flucloxacillin	0.015 mg/L
	Doxycycline	0.063 mg/L

MIC values represent the consistent result obtained across three biological replicates (N=3), each performed with two technical replicates, with no variation observed between experiments.

Based on the MICs for each antibiotic, certain antibiotic concentrations were chosen for further testing. Since the MICs of *S. aureus* and *S. pyogenes* were much lower than that *of E. coli* for all three antibiotics, the concentration range chosen for each antibiotic was dependent on the MICs of *S. aureus* and *S. pyogenes*. Based on the initial testing of penicillin exposure in the two-species model, sub-MIC and above-MIC levels of antibiotic exposure were chosen (see Table 3.5). Each antibiotic was tested at the following concentrations -

(a) sub-MIC (0.5xMIC) of *S. pyogenes*

(b) sub-MIC (0.5xMIC) of *S. aureus,* and

(c) above-MIC (4xMIC) of *S. aureus*.

In the case of penicillin and flucloxacillin, since the MIC of *S. aureus* was greater than that of *S. pyogenes*, concentrations (b) and (c) also allowed the testing of the three-species model at concentrations that were above-MIC with respect to *S. pyogenes*. In the case of doxycycline, the MIC of *S. aureus* and *S. pyogenes* were equal, and thus enabled the three-species model to be tested at sub- and above-MIC levels with respect to both strains.

Table 3.5: Antibiotic concentrations tested in the three-species model and their relationship to MICs of S. pyogenes and S. aureus

Antibiotic	**Concentrations Tested**	***S. pyogenes***	***S. aureus***
Penicillin	- 0.008 mg/L	0.5xMIC	sub-MIC
	- 0.03 mg/L	above-MIC	0.5xMIC
	- 0.24 mg/L	above-MIC	4xMIC
Flucloxacillin	- 0.008 mg/L	0.5xMIC	sub-MIC
	- 0.063 mg/L	above-MIC	0.5xMIC
	- 0.5 mg/L	above-MIC	4xMIC
Doxycycline	- 0.03 mg/L	0.5xMIC	0.5xMIC
	- 0.24 mg/L	4xMIC	4xMIC

The three-species model was tested in the presence of these antibiotics and evaluated for survival in single-species cultures in comparison to mixed-species cultures.

3.4.1.4 *A. baumannii* and *E. coli* produce a β-lactamase allowing for growth of *S. aureus* in a modified Hodge test

A modified Hodge test was performed to detect the production of β-lactamases by organisms confirmed to be penicillin resistant (*A. baumannii* and *E. coli* strains). A cloverleaf-like indentation (Figure 3.4) was observed at the intersection of the sensitive strain, *S. aureus*, and the resistant strains, *A. baumannii* and *E. coli.* This indicated that *A. baumannii* and *E. coli* were β-lactamase producers, inactivating the penicillin closer to the disc in the centre and allowing the sensitive strain to grow in that zone.

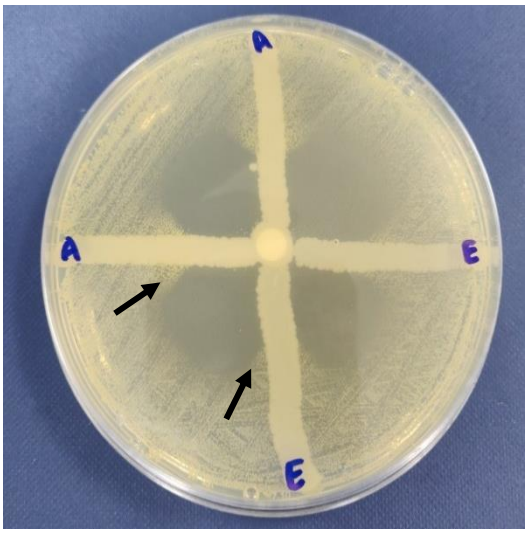

Figure 3.4: Modified Hodge Test*. This shows a cloverleaf like indentation (arrows). The uniform growth on the plate represents S. aureus. The lines of bacterial growth labelled A represent A. baumannii and those labelled E represent E.coli growth (N=3).*

3.4.2 Exploration of mixed community dynamics in a two-species model

3.4.2.1 Population dynamics vary in mixed communities compared to single-species cultures

S. aureus and *A. baumannii* exhibited different patterns in endpoint viable population densities in single-species and mixed communities in planktonic and biofilm cultures (Figure 3.5). In single-species planktonic culture, after 24 hours, the endpoint viable density of *S. aureus was* higher than *A. baumannii* (p=0.03), though this difference was less than 1-log. In planktonic mixed-species communities, *S. aureus* demonstrated significantly higher endpoint viable counts than *A. baumannii*

(p<0.0001). Conversely, in the biofilm culture model, a reversal of growth patterns was observed. Endpoint viable counts of *A. baumannii* were significantly higher than *S. aureus* in both single-species and mixed-species biofilms (p<0.001 and p<0.05 respectively). While *S. aureus* grew similarly in both single- and mixed-species communities, *A. baumannii* had significantly less growth in the mixed communities in both planktonic and biofilm cultures compared to single-species communities (p<0.0001 and p<0.01 respectively).

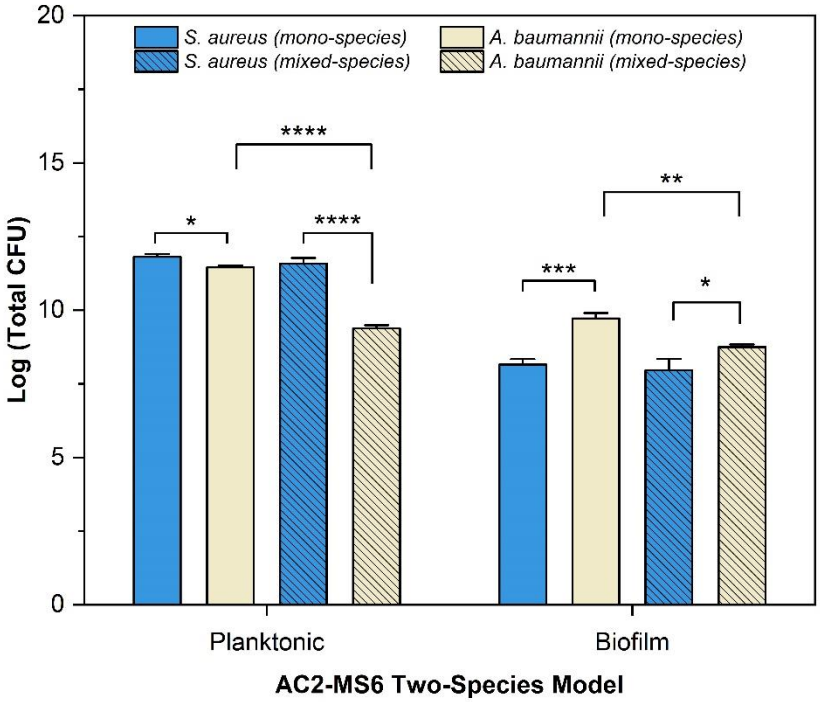

Figure 3.5: Population dynamics of the AC2-MS6 model. *Log value of total CFU per culture. Bars represent mean (±SD) values, N=3, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001*

A two-species model with *S. aureus* and *E. coli* also exhibited differential population dynamics (Figure 3.6). In planktonic single-species cultures, the endpoint viable population of *E. coli* was significantly less than *S. aureus* (p<0.0001), however, this trend was reversed in the mixed-species cultures (p<0.0001). Additionally, both strains exhibited reduced endpoint viable populations in planktonic mixed communities compared to their respective single-species planktonic cultures, and *S. aureus* had a similar trend in biofilms.

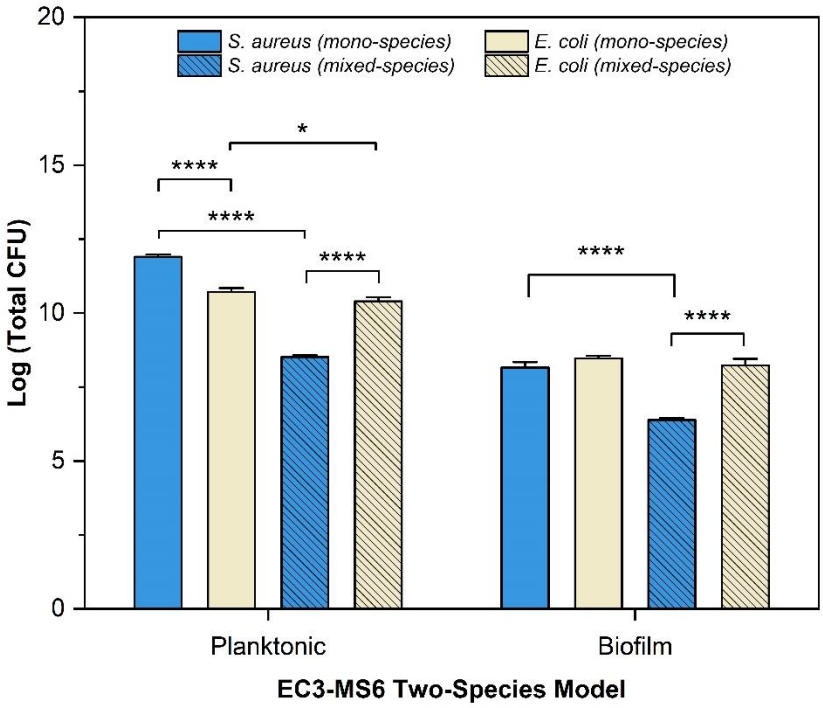

Figure 3.6: Population dynamics of the EC3-MS6 model. *Log value of total CFU per culture. Bars represent mean (±SD) values, N=3, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001*

3.4.2.2 Resistant strains provide a protective effect to sensitive species against penicillin in dual-species model

To assess the impact of antibiotics on survival within mixed-species biofilms communities, dual-species populations containing *A. baumannii* + *S. aureus* and *E. coli* + *S. aureus* combinations were grown in the presence of penicillin at two sub-MIC concentrations (antibiotic added at 0h time point based on the MIC of *S. aureus*) (Figure 3.7). *S. aureus* single-species biofilms exhibited a reduced survival ratio when grown in the presence of 0.03 mg/L ($p<0.001$) and 0.04 mg/L ($p<0.0001$) penicillin compared to those grown in the presence of no antibiotic. Growth of *A. baumannii* single-species biofilms was unaffected by penicillin (Figure 3.7A). For *A. baumannii* + *S. aureus* mixed-species biofilms which were grown in the presence of 0.03 mg/L penicillin, an increased survival ratio for *S. aureus (*when compared to its single-species biofilms at the same antibiotic concentration) was observed ($p<0.0001$) (Figure 3.7A). A similar effect was also observed at 0.04 mg/L penicillin. The survival ratio of *A. baumannii* in mixed-species biofilms at 0.03 mg/L was slightly increased in

comparison to that in single-species at the same antibiotic concentration, but the same effect was not seen at 0.04 mg/L penicillin.

In the *E. coli* + *S. aureus* mixed-species biofilm model, a similar protective effect was seen in mixed-species biofilms (Figure 3.7B). *S. aureus* exhibited a higher survival ratio in mixed communities compared to its single-species biofilms at 0.03 mg/L and 0.04 mg/L penicillin concentrations ($p<0.05$ and $p<0.01$ respectively). The survival of *E. coli* was unaffected by either the presence of penicillin or co-culture with *S. aureus*.

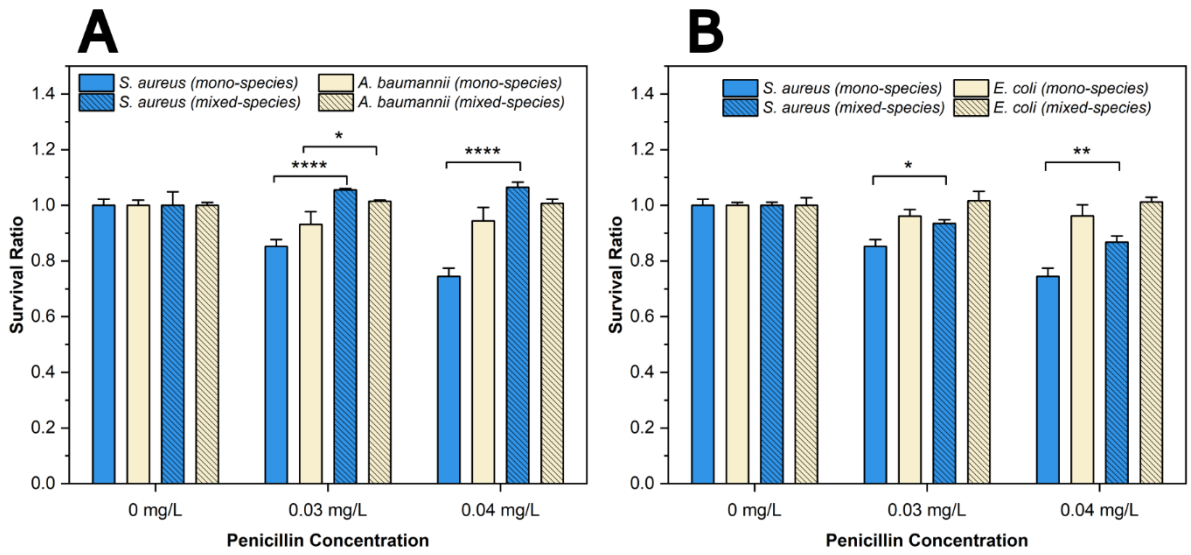

Figure 3.7: Survival ratio in two-species biofilm cultures in the presence of penicillin. *Data was normalised to 0mg/L using the survival ratio equation. (A) S. aureus + A. baumannii Two-species biofilm model (B) S. aureus + E. coli Two-species biofilm model. Bars represent mean (±SD) values, N=3, * p<0.05, ** p<0.01, **** p<0.0001*

Exposure of these dual-species biofilm models to penicillin showed a protective effect of the resistant strains towards the sensitive strain. While both dual-species models showed an increased survival ratio of the penicillin sensitive strain, the *A. baumannii* + *S. aureus* co-culture exhibited additional marginal effects on survival of the resistant strain. To eliminate this additional variable, the *E. coli* + *S. aureus* model was taken forward for further investigations, and an *S. pyogenes* strain was introduced to generate a three-species model.

3.4.3 Exploration of mixed community dynamics in a three-species model

3.4.3.1 *S. aureus, E. coli* and *S. pyogenes* exhibit differential growth from each other in both planktonic and biofilm communities

S. aureus, *E. coli* and *S. pyogenes* were grown as single- and mixed-species communities in planktonic (Figure 3.8A) and biofilm (Figure 3.8B) cultures. In planktonic single-species cultures, *S. aureus* and *E. coli* exhibited similar log-values of total CFU per culture (9.68± 0.13 and 9.89±0.03), whereas *S. pyogenes* has slightly lower growth than both (9.13±0.22) (Figure 3.8A). In a mixed-planktonic culture, both *S. aureus* (8.97±0.04) and *S. pyogenes* (8.84±0.14) had lower growth than *E. coli* (9.84±0.06) in the mixed community. *S. aureus* had less growth in mixed communities compared to the single-species planktonic cultures ($p<0.001$).

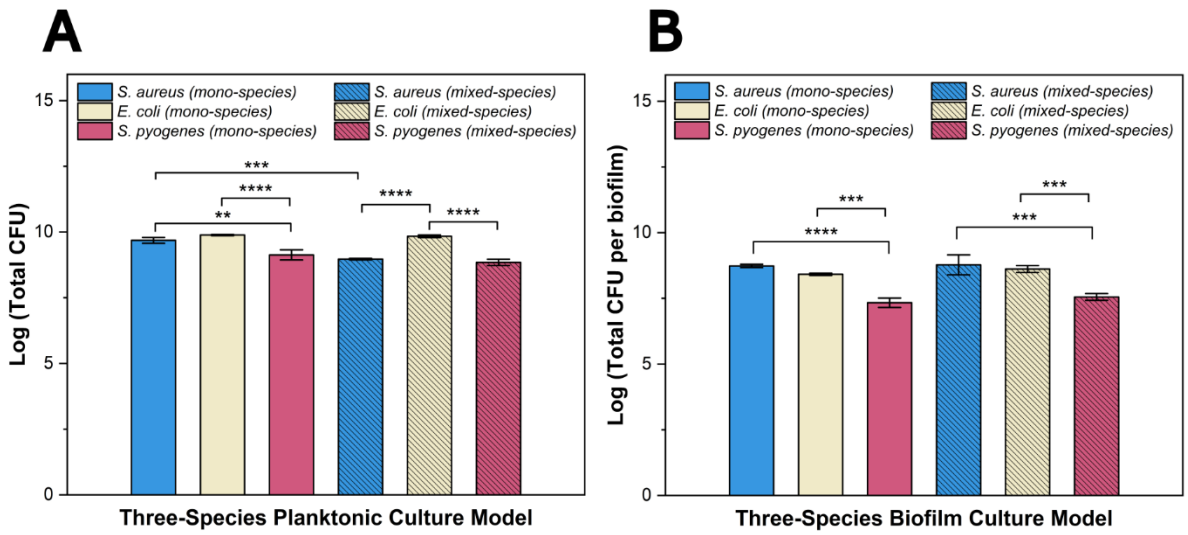

Figure 3.8: Population dynamics of the three-species model after 24 hours. *Log(Total CFU) values for the three species model in (A) planktonic and (B) biofilm single-species and mixed-species cultures. In planktonic cultures, S. aureus and E. coli exhibited similar CFU values in single-species cultures, while S. pyogenes showed lower growth. In mixed-species planktonic cultures, both S. aureus and S. pyogenes demonstrated reduced growth compared to E. coli. A slight reduction in CFU values was noted for S. aureus in mixed-species compared to single-species planktonic cultures. For biofilms, S. pyogenes exhibited lower CFU values than S. aureus and E. coli in both single- and mixed-species conditions. No significant differences were observed between single- and mixed-species biofilms for any species. Bars represent mean (±SD) values, N=3, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001*

S. pyogenes also had a lower log (Total CFU) per single-species biofilm (7.33±0.21) than *S. aureus* (8.73±0.07) and *E. coli* (8.42±0.05) (Figure 3.8B). A similar population dynamic was observed in the mixed-species biofilms, with *S. pyogenes* present at 7.55±0.15 log (total CFU) per biofilm, and *S. aureus* and *E. coli* colony counts being higher (8.77±0.44 and 8.62±0.15). No significant difference in growth was seen between single- and mixed-species biofilms for *S. aureus, E. coli* and *S. pyogenes* (p=0.99, p=0.86 and p=0.82 respectively).

3.4.3.2 Scanning electron microscopy revealed single- and mixed-species cultures attach and form biofilms on the fine-celled foam substrate

Scanning electron microscopy revealed that all three strains, *S. aureus, E. coli* and *S. pyogenes*, attached to and formed biofilms on the fine-celled foam substrate (Figure 3.9). The substrates showed regions of both high and low bacterial densities. *S. aureus* depicted typical cocci in cluster-like arrangements, while *E. coli* depicted bacilli as expected, and *S. pyogenes* also showed characteristic chain-like arrangements (Klainer & Betsch, 1970). The substrates went through multiple washes, and hence the presence of bacterial communities showcases attachment to the substrate. Bacterial communities were also seen enveloped in a layer (see black arrows in Figure 3.9), likely the biofilm matrix, further supporting that biofilms form on this substrate. Mixed-species biofilms showed the presence of both cocci and bacilli. A closer look at morphology and arrangement enabled the identification of *S. aureus* (arrow 1), *E. coli* (arrow 2) and *S. pyogenes* (arrow 3) within mixed-species biofilms, thus showcasing that the three species are present in proximity within these biofilms.

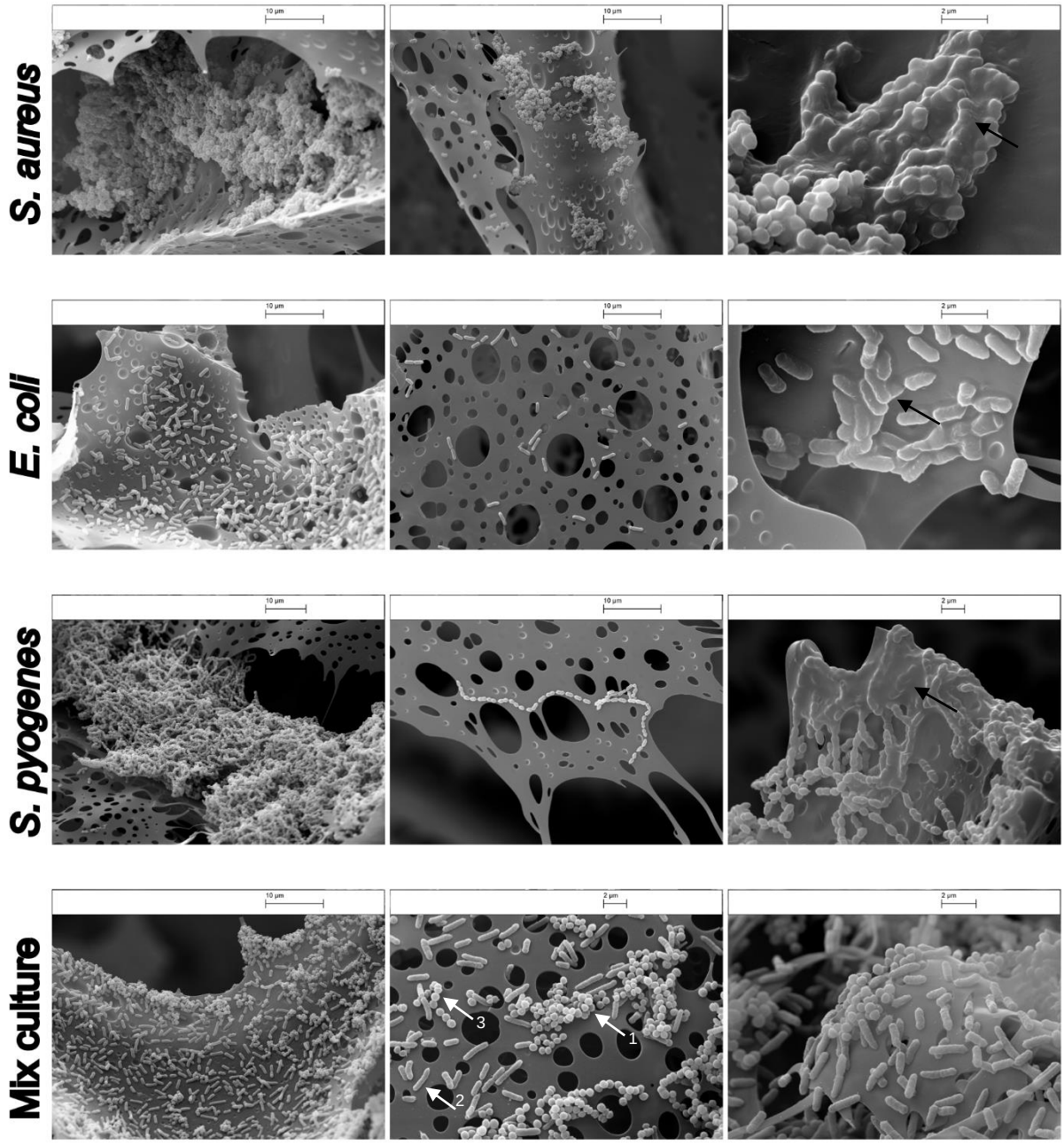

Figure 3.9: Scanning electron microscopy of single and mixed-species biofilms. *Single-species biofilms of S. aureus, E. coli, S. pyogenes and mixed-species show attachment of bacteria on the fine-celled foam and biofilm formation.*

3.4.3.3 Three-species planktonic and biofilm communities show protective effects in the presence of penicillin

The three-species model was assessed for growth in the presence of different concentrations of penicillin in planktonic (Figure 3.10A) and biofilm (Figure 3.10B) communities.

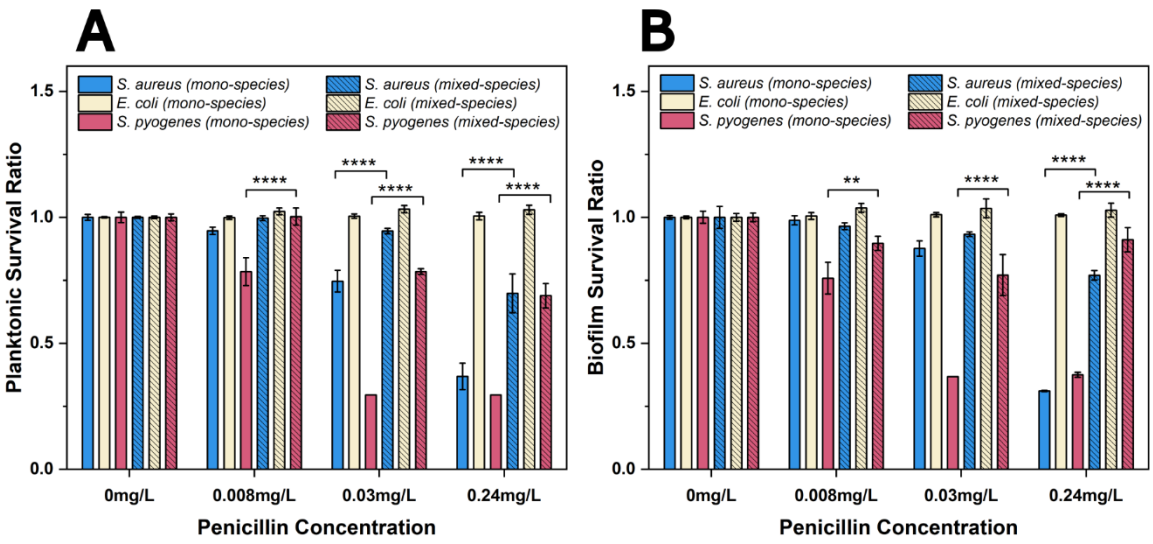

Figure 3.10: Survival ratio of the three-species model in the presence of penicillin. *Survival ratios of the three species were assessed after 24 hours of exposure to different concentrations of penicillin in (A) Planktonic and (B) Biofilm cultures. In planktonic cultures, S. pyogenes showed significantly higher survival in mixed-species communities compared to single-species communities at all tested concentrations. A similar protective effect was observed for S. aureus at 0.03 mg/L and 0.24 mg/L. In biofilms, S. pyogenes maintained increased survival in mixed-species communities at all concentrations of penicillin tested, whereas S. aureus showed increased survival only at 0.24 mg/L. E. coli survival remained unaffected across all concentrations in planktonic and biofilm cultures. Bars represent mean (±SD) values, N=3, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001*

At 0.008 mg/L penicillin, *S. pyogenes* demonstrated a significantly higher survival ratio in the planktonic mixed-species communities ($p<0.0001$) compared to the single-species communities. The survival ratio was found to be 1.003±0.040, indicating that in mixed-species planktonic communities, survival of *S. pyogenes* was similar to that in mixed-species communities with no antibiotic, suggesting a strong protective community effect. At both 0.03 mg/L and 0.24 mg/L, a similar protective effect was seen, with a significantly higher survival ratio observed in mixed-species communities

compared to mono-species communities for both *S. pyogenes* and *S. aureus* (Figure 3.10A). However, since 0.03 mg/L and 0.24 mg/L represent above-MIC concentrations with respect to *S. pyogenes*, the survival ratios in mixed-species planktonic cultures were <1, suggesting that the survival of *S. pyogenes* in mixed-species cultures at these concentrations was still significantly reduced from that in mixed-species cultures without antibiotic ($p<0.001$). A similar trend was observed for *S. aureus* in mixed-species cultures at 0.24 mg/L ($p<0.001$). *E. coli* remained unaffected in both planktonic and biofilm cultures within the range of the antibiotic tested.

In the mixed-species biofilms, *S. pyogenes* exhibited a similar pattern of increased survival ratio compared to mono-species biofilms at all concentrations of penicillin tested (Figure 3.10B). While *S. aureus* did exhibit a higher survival ratio at 0.03 and 0.24 mg/L in planktonic mixed communities (Figure 3.10A), it only exhibited this increased survival ratio at 0.24 mg/L in mixed-species biofilms (Figure 3.10B).

3.4.3.4 Three-species model shows protective effects in the presence of flucloxacillin

The three-species model was also tested in the presence of a range of flucloxacillin concentrations in planktonic (Figure 3.11A) and biofilm (Figure 3.11B) cultures. Interestingly, at 0.008 mg/L flucloxacillin, no differences were seen in mono- and mixed-species planktonic cultures (Figure 3.11A) for all three bacterial strains. *S. pyogenes* exhibited a higher survival ratio in mixed-species planktonic communities compared to mono-species communities at both 0.063 mg/L and 0.5mg/L flucloxacillin ($p<0.0001$), though the extent of this effect was seen to be concentration dependent. At a higher concentration of flucloxacillin, the difference between survival ratios of mixed- and mono-species cultures was lower, likely due to the higher levels of antibiotic resulting in more bacterial killing. While *S. aureus* had survival ratios of <1 in both mono- and mixed-species cultures at 0.063 mg/L flucloxacillin, there was no significant difference between mono- and mixed-species planktonic communities. A higher survival ratio was observed for *S. aureus* in mixed-species planktonic cultures at 0.5 mg/L compared to mono-species cultures ($p<0.0001$); however, this protective effect only managed to restore growth to ~50% of that observed in mixed-species

without antibiotic (survival ratio of 0.501±0.016). *E. coli* remained unaffected in both planktonic and biofilm cultures within the tested range of antibiotics.

The effects of mixed-species in comparison to mono-species biofilms exposed to flucloxacillin (Figure 3.11B) were found to be species dependent, unlike what was observed for biofilms grown in the presence of penicillin. *S. aureus* showed a lower survival ratio in mixed-species biofilms compared to single-species biofilms at 0.008 mg/L and 0.0063 mg/L ($p<0.0001$). Only at above-MIC concentrations (0.5 mg/L) was a slight protective effect observed in mixed-species biofilms ($p<0.0001$), which increased the survival ratio to 0.489±0.007. *S. pyogenes* exhibited an increased survival ratio in mixed-species biofilms compared to single-species biofilms at all concentrations tested.

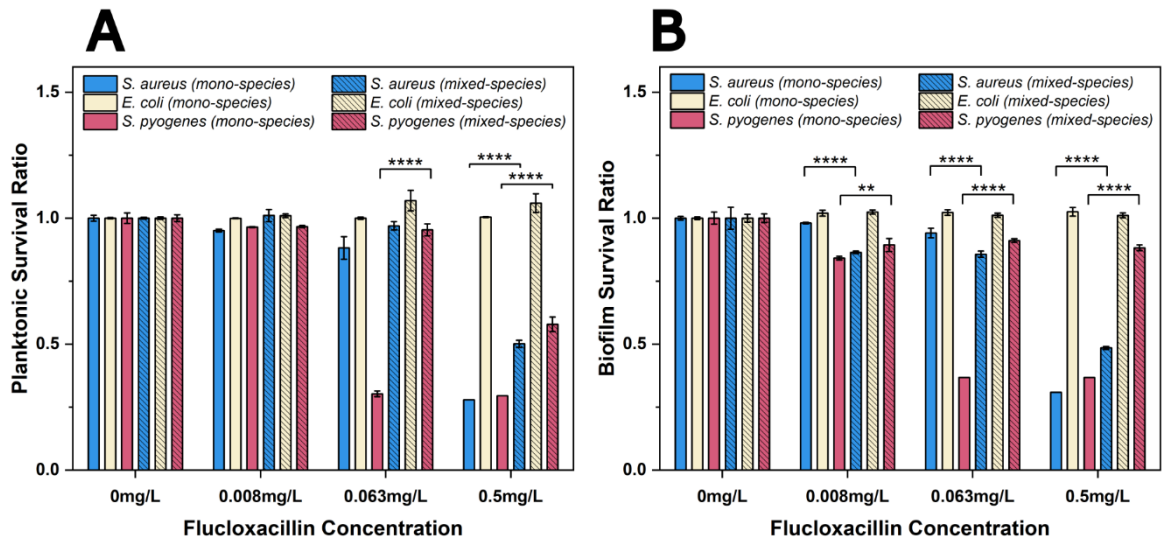

Figure 3.11: Survival ratio of the three-species model in the presence of flucloxacillin. *Survival ratios of the three species were assessed after 24 hours of exposure to varying concentrations of flucloxacillin in (A) Planktonic and (B) Biofilm cultures. S. pyogenes showed higher survival in mixed-species planktonic and biofilm cultures compared to mono-species cultures at all concentrations (except 0.008 mg/L planktonic cultures). S. aureus exhibited a higher survival in mixed-species communities compared to mono-species at 0.5 mg/L in both planktonic and biofilm cultures. E. coli survival was unaffected in all cultures and all antibiotic concentrations tested. Bars represent mean (±SD) values, N=3, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001*

3.4.3.5 Three-species model exhibited slight protective effects in the presence of doxycycline only at concentrations above MIC

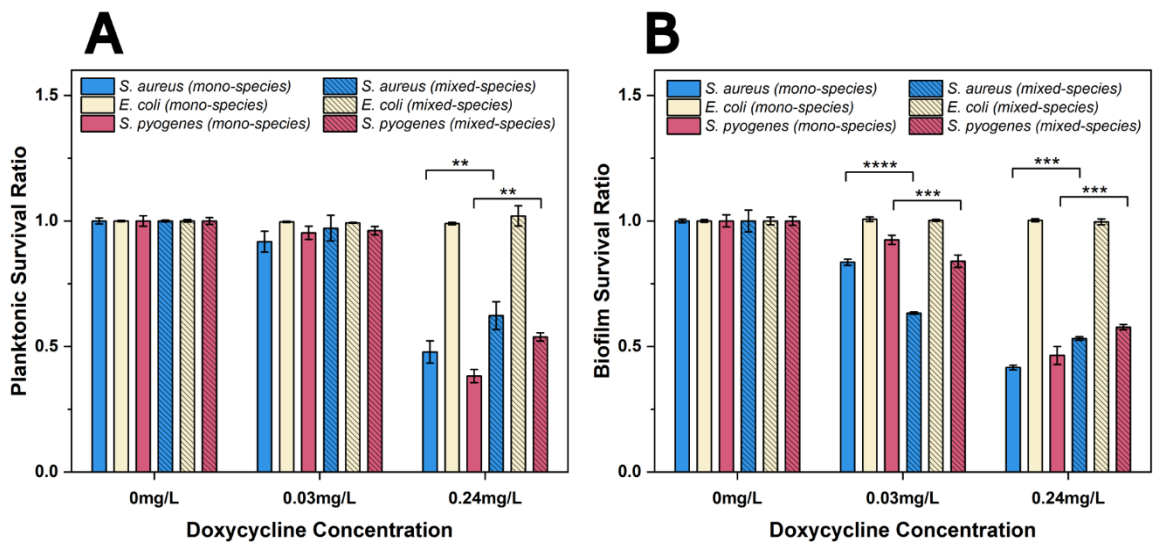

Figure 3.12: Survival ratio of the three-species model in the presence of doxycycline. *The three-species model was grown in the presence of doxycycline for 24 hours and survival ratio of each species was evaluated in (A) Planktonic and (B) Biofilm cultures. At 0.24 mg/L doxycycline, S. aureus and S. pyogenes showed slightly higher survival in mixed-species communities compared to mono-species communities, but still experienced significant reductions compared to growth without the antibiotic. E. coli survival remained unaffected in all conditions. Bars represent mean (±SD) values, N=3, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001*

When the three-species planktonic model was exposed to a sub-MIC level (0.03 mg/L) of doxycycline, no differences were observed between mono- and mixed-species cultures for all three bacterial species (Figure 3.12A). However, *S. aureus* and *S. pyogenes* exhibited lowered survival ratios in mixed-species biofilms compared to single-species biofilms at the same concentration of doxycycline (Figure 3.12B), highlighting differences between planktonic and biofilm states for bacteria. At above-MIC levels (0.24 mg/L), a slight protective effect was seen in mixed-species communities compared to single-species communities for both *S. aureus* and *S. pyogenes* in planktonic (Figure 3.12A) and biofilm (Figure 3.12B) cultures. However, the mixed communities at 0.24 mg/L doxycycline were able to rescue growth of both *S. aureus* and *S. pyogenes* only to ~50% of that in mixed communities without the antibiotic, indicating significant killing of the strains even in mixed communities at this high antibiotic concentration. *S. aureus* survival ratio in planktonic cultures at 0.24

mg/L increased from 0.478±0.051 in mono-species cultures to 0.623±0.063 in mixed-species cultures. In biofilm communities, this difference was even lower, with mono-species *S. aureus* biofilms exhibiting a survival ratio of 0.416±0.011 and *S. aureus* in mixed-species biofilms exhibiting a survival ratio of 0.532±0.009 (Figure 3.12B). Similarly, the survival ratio of *S. pyogenes* increased from 0.382±0.031 in mono-species planktonic cultures to 0.538±0.019 in mixed-species planktonic cultures (Figure 3.12A). In biofilm communities, the survival ratio of *S. pyogenes* increased from 0.464±0.041 in mono-species biofilms to 0.577±0.012 in mixed-species biofilms (Figure 3.12B). *E. coli* survival remained unaffected in both planktonic and biofilm mono- and mixed-species communities.

3.5 Discussion

The polymicrobial nature of wound infections can present a significant challenge. As the entire microbial community is exposed to antibiotic therapy different members of these mixed-species communities may influence the efficacy and thus the overall treatment outcomes and wound healing. *In vitro* mixed-species bacterial community models can shed light on their population dynamics and provide further insights into these communities in the context of antibiotic recalcitrance. This study utilised an *in vitro* model to assess the effects of antibiotic exposure on the population survival dynamics of three-species communities.

When selecting and utilising the *in vitro* model, it was essential to consider a system that facilitates the study of population dynamics in both planktonic and biofilm states. Biofilm communities are particularly significant in wound infections (Cooper et al., 2014; James et al., 2008; Malone et al., 2017a; Percival et al., 2015). Therefore, incorporating a biofilm component into the model was crucial for accurately reflecting the complexity and challenges of treating such infections. While other biofilm models incorporate flow conditions (Vyas et al., 2022), the use of a static model has various advantages. Static models are generally easier to set up, are cost-effective and require less specialised equipment compared to flow models, which often need pumps, tubing, and flow chambers. Static models also provide a highly controlled environment, where variables can be kept consistent across multiple experiments, making it easier to reproduce results. Static models are high throughput, allowing multiple conditions to be tested simultaneously, as well as easy to replicate. However, static models also have limitations including lack of fluid dynamics, simplified environment and surface limitations. Despite these limitations, static models can provide insights into biofilm formation and antibiotic susceptibility in relatively shorter time frames, making them suitable for research.

In previous studies, the substrate of choice for biofilm growth has varied, ranging from simple well-plates to more complex collagen scaffolds (Brackman & Coenye, 2016; Guzman-Soto et al., 2021; Thaarup & Bjarnsholt, 2021; Thaarup et al., 2023; Vyas et al., 2022). In this study, a fine celled phenolic foam was used as the substrate for biofilm growth. This substrate has been shown previously to support bacterial attachment and biofilm formation and has features such as the ability to undergo heat

sterilisation and liquid absorption and maintain structural integrity, and presence of pores that facilitate uptake of liquid (Oates & McBain, 2016). Attachment to the substrate was confirmed using scanning electron microscopy, wherein all three bacterial strains of the model, *S. aureus, E. coli,* and *S. pyogenes*, successfully attached to and formed biofilms on the substrate. The observation of bacterial communities enveloped in a matrix-like layer further supports biofilm formation on this substrate. This finding is crucial as it further validates the suitability of the fine-celled foam for studying biofilm dynamics *in vitro*.

To validate the model for growth dynamics and to identify if mixed communities have any effects on survival upon antibiotic exposure, a preliminary two-species model was developed, using three different strains – *S. aureus, A. baumannii* and *E. coli* – and one β-lactam antibiotic (benzylpenicillin). *S. aureus* has been implicated as a key pathogen in wound infections and thus was a relevant choice. *A. baumannii* has been implicated in skin and soft tissue infections (Castellanos et al., 2019; Guerrero et al., 2010) and Malone et al. (2017b) found its prevalence to be ~30% in infected diabetic tissue samples. *E. coli* has also been identified in wound communities, with a particular meta-analysis of 112 datasets identifying it as one of the top three organisms frequently isolated from diabetic foot infections (Macdonald et al., 2021). The pilot phase of this study explored the population survival dynamics of dual-species models of *A. baumannii* + *S. aureus* and *E. coli* + *S. aureus* with and without antibiotic.

Data from the two-species model suggest survival dynamics are both species and lifestyle dependant. This in part could be explained by the different growth rates of the strains (Figure 3.1) as well as a competition for nutrients and space. Since *S. aureus* had a higher growth rate than *A. baumannii*, in planktonic co-cultures it was able to grow faster, outcompeting *A. baumannii* for nutrients and space. This contrasts with a previous study which evaluated *A. baumannii* and *S. aureus in vitro* co-culture using strains isolated from skin and soft tissue of a diabetic patient (Castellanos et al., 2019). The authors found no significant difference in growth when the two species were co-cultured, which contrasts with the findings of this study, where *S. aureus* displayed higher growth in planktonic co-culture. However, this is expected as the strains and model set-up are different in comparison to the current study. In the case of *A. baumannii* and *S. aureus* biofilms, where substrate attachment likely played a key role,

the trend was reversed. This further highlights the importance of studying the biofilm lifestyle when developing *in vitro* models.

In dual-planktonic cultures with *E. coli*, *S. aureus* likely competed for limited nutrients with its lower growth rate. This illustrates the importance of considering species-specific interactions and growth dynamics when studying microbial communities. It highlights how the presence of different bacterial species can significantly influence population outcomes due to factors like growth rates and competition for resources.

Using the two-species model, this study aimed to explore the potential protective effect of β-lactamase production by β-lactamase producers on the survival of β-lactam sensitive strains with penicillin selective pressure. Penicillins are β-lactam antibiotics. Consisting of a β-lactam ring, penicillins work by inhibiting cross-linking of peptidoglycan in the cell wall, thus preventing cell wall synthesis, leading to bacterial death. Penicillin resistance has been a long-known problem (Abraham & Chain, 1988; Lobanovska & Pilla, 2017), with bacteria evolving enzymes that can degrade the drug or modifying the structure of the target (penicillin-binding proteins) (Reygaert, 2018). In this study, dual-species biofilms containing a β-lactamase producing strain showed a protective effect towards the penicillin-sensitive strain (*S. aureus*) when exposed to benzylpenicillin. The core component of this effect is likely the degradation of the β-lactam antibiotic by the β-lactamase. Thus, in the presence of a β-lactamase producing strain, the overall antibiotic amount reduces in the environment, providing 'protection' to sensitive strains in the same region. However, the extent of the protective effect observed herein was dependent on the resistance profile of the strains and likely influenced by factors such as competition for nutrients and space. When multiple species coexist in a limited environment, they must compete for essential resources such as carbon sources, nitrogen, and other growth factors. This competition can impact the growth rates and metabolic activity of each species. Additionally, the physical space within the biofilm is another limited resource, with bacteria competing for attachment sites and biofilm structure. For example, when co-cultured with *A. baumannii*, the protective effect was able to restore the viable populations of *S. aureus* to similar levels as those seen in co-cultures without antibiotic. However, this was not the case with *E. coli* co-culture biofilms, likely due to differences in growth rates, attachment and competition for nutrients. Despite this, the

protective effect was significant in *E. coli* + *S. aureus* biofilms. This ‘protective effect’ has been observed before with different microbial species combinations. For example, an imipenem-sensitive strain of *P. aeruginosa* experienced a protective effect from a multi-drug resistant β-lactamase producer *S. maltophilia* when their co-culture was exposed to imipenem (Bottery et al., 2022). Another study by the same group also showed that in such co-cultures, *P. aeruginosa* evolved resistance to imipenem at an increased rate (Quinn et al., 2022). However, this in turn negatively affected viable cell density levels of S*. maltophilia* in the co-culture, likely due to a metabolic cost of enzyme production, suggesting that the relationship is not mutualistic. In another study, a β-lactamase producing *Prevotella* isolate provided a similar protective effect to a β-lactamase negative *P. aeruginosa* in the presence of ceftazidime at 64 times the MIC of *P. aeruginosa* (Sherrard et al., 2016). Previous findings from various co-culture studies in the presence of β-lactams, alongside the findings of the dual-species model in this study, underscore the intricate interactions within mixed-species communities, which can significantly influence both protective mechanisms and population dynamics within the communities upon exposure to this group of antibiotics.

Overall data for the two-species model supported the hypothesis that β-lactamase production can provide a protective effect to other organisms when under a β-lactam selective pressure. Building on these findings seen with the *E. coli* + *S. aureus* combination, a three-species community model was developed, incorporating *S. pyogenes* as the third member of the community. *Streptococcus* spp. have been frequently found in wounds (Candel Gonzalez et al., 2003; Dorr et al., 2021; Gardner et al., 2001; Macdonald et al., 2021) and *S. pyogenes* has been isolated from skin and soft tissue infections as well as diabetic foot infections (Citron et al., 2007; Lamagni et al., 2008a; Lamagni et al., 2008b). One of the key aspects of this study is the expansion of both the number of organisms and the range of antibiotics tested, moving beyond just β-lactams. By including an additional bacterial species and testing with three different antibiotics, this study has demonstrated that these protective interactions are not limited to β-lactamase-mediated resistance. This approach provides a more comprehensive understanding of how microbial communities interact and respond to various antibiotic pressures, emphasising the importance of considering multiple factors and interactions when studying antibiotic resistance in mixed-species communities.

While the preliminary work with the two-species model indicated a protective effect in the presence of penicillin, this effect can also be seen in a three-species community. The protective effect was seen to be dependent on the concentration of drug the community was exposed to, as well as the MIC of the sensitive strains. While *S. pyogenes* consistently experienced this protective effect in planktonic and biofilm mixed communities, *S. aureus* only experienced the effect at above-MIC concentration in biofilms. This highlights the complex nature of biofilms and mixed communities, where the drug concentration is likely not the only aspect influencing population dynamics.

Alongside benzylpenicillin, flucloxacillin and doxycycline were also evaluated in the three-species model. Flucloxacillin belongs to the penicillin group and thus is bactericidal, affecting cell wall synthesis, and is the first antibiotic of choice when treating diabetic foot infections (National Institute for Health and Care Excellence - NICE guideline). Flucloxacillin is different from benzylpenicillin in structure with an acyl side chain attached to the β-lactam ring, which prevents penicillinase from accessing the ring and degrading the drug, thus making flucloxacillin effective in the case of infections with penicillinase producers. Flucloxacillin is a narrow-spectrum antibiotic, usually prescribed against Gram-positive bacterial infections. Doxycycline is also another drug of choice when treating diabetic foot infections, especially when the patient has a penicillin allergy or if flucloxacillin is not suitable (National Institute for Health and Care Excellence - NICE guideline). Doxycycline belongs to the tetracycline group of antibiotics and is a bacteriostatic, binding to the 30S ribosomal subunit and preventing protein synthesis. According to the English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR) report of 2022-23 (UK Health Security Agency, 2023), both doxycycline and flucloxacillin were part of the top widely used antibiotics in the UK. Hence their inclusion in this study provides novel insights into population dynamics in mixed-species communities in the presence of these key wound infection therapies.

When the three-species model was exposed to varying concentrations of flucloxacillin, a protective effect was observed for both *S. pyogenes* and *S. aureus* in planktonic and biofilm mixed communities at high drug concentrations. This finding is particularly noteworthy as *S. aureus* wound infections are commonly treated with flucloxacillin.

Clinical culture reports typically focus on the predominant species as the likely causative organisms, potentially overlooking the presence and impact of Gram-negative bacteria such as *E. coli*. Therefore, in cases where *S. aureus* is identified as the causative pathogen, the presence of Gram-negative bacteria like *E. coli* in the wound microbiota could influence treatment outcomes, especially if they provide a protective effect as seen in this study. The protective effect for high concentrations of flucloxacillin seen herein is supported by the findings of Hill et al. (2010), where exposure of mixed-species biofilms to flucloxacillin at above-MIC concentration resulted in no reduction of viable counts for up to 5 days of antibiotic treatment. It is important to note that some variable effects were also observed, such as the slight reduction in *S. aureus* survival in mixed-biofilms at low concentrations of the drug, a phenomenon not seen in planktonic cultures. It is hypothesised that this effect, observed only at sub-MIC flucloxacillin concentrations in biofilms, is due to the complex biofilm architecture, microenvironmental factors, and interspecies interactions affecting drug penetration, nutrient competition, and stress responses.

Interestingly, the current study also showed that the 'protective' effect of mixed communities is not limited to β-lactams such as penicillin and flucloxacillin, but is also seen in the non-β-lactam, bacteriostatic antibiotic - doxycycline. At the highest doxycycline concentration, viable counts of *S. aureus* and *S. pyogenes* in mixed communities recovered to ~50% of that observed in the presence of no antibiotic. In contrast, mixed-species biofilms at a lower doxycycline concentration (0.03 mg/L) showed reduced viable counts than single-species biofilms. Whilst the levels of viable populations of *S. aureus* and *S. pyogenes* at high doxycycline concentrations in both planktonic and biofilm communities did not reach the same protected levels observed with benzylpenicillin and flucloxacillin, there is evidence of a significant protective effect. This suggests that factors beyond enzymatic antimicrobial destruction are at play. A plausible explanation for this phenomenon is that the total cell densities were higher in mixed communities compared to monocultures. This indicates that likely a greater number of cells collectively take up the antimicrobial agent, reducing the concentration of the active compound available to each cell. This effect was only seen at concentrations above the MIC (specifically at four times the MIC) and not at sub-MIC levels. At sub-MIC concentrations, the antibiotic might not be present in high enough amounts to be significantly depleted by collective uptake, while at higher

concentrations, the microbial community can effectively reduce the available antibiotic, thus demonstrating a protective effect. However, there could also be other factors responsible for this protective effect. It is plausible that the antibiotic pressure could result in interspecies interactions that may facilitate improved stress response, altered metabolism, increased efflux pump activity and changes in gene expression.

Interestingly at sub-MIC levels of doxycycline, planktonic communities survived at similar levels to those seen for antibiotic free controls, and there was no evidence of a protective effect. However, in sessile mixed communities, the number of viable cells of *S. aureus* and *S. pyogenes* were lower than their mono-species counterpart. Additionally, for *S. aureus*, the survival ratio of even the mono-species biofilm was lower than that at antibiotic-free conditions. This is unexpected because the biofilm lifestyle is typically associated with recalcitrance to antimicrobials due to several factors, including reduced metabolic activity of organisms, the presence of dormant cells, and limited penetration of the antibiotic through the biofilm matrix. These characteristics usually make biofilms have higher levels of recalcitrance to antibiotic treatment. However, that was not observed in this case at sub-MIC levels. One possibility is that in mixed-species biofilms, there may be increased competition for limited resources such as nutrients and space, which could lead to stress and reduced viability of certain species. This interspecies competition could offset the typical protective benefits of the biofilm lifestyle at sub-MIC levels. Furthermore, factors like metabolic activity, stress responses, altered gene expression in biofilms can also contribute to this observed effect.

Overall, this study sheds light on the importance of studying mixed communities *in vitro*, in both planktonic and biofilm states. The differences in population survival observed in biofilms, in contrast to those in planktonic states, support the vast evidence in literature that bacteria in biofilms have a different ‘lifestyle’ than their planktonic counterparts. Bacteria within biofilms exacerbate the problem of antibiotic resistance (Bowler et al., 2020; Mah & O'Toole, 2001), as they have different phenotypes and metabolism, show increased horizontal transfer and gene uptake and can be up to 1000 times more resistant than planktonic bacteria. Nevertheless, data herein show that this phenomenon may not be ubiquitous. Specifically, some data,

such as that for doxycycline, indicate that in a mixed community biofilm, members of the communities may be more susceptible to antibiotics.

While this study showcases the protective effect mixed communities can offer sensitive strains, using a rapid, reproducible static model, there are some inherent limitations with this approach. The utilisation of standardised laboratory broth culture media was useful in understanding the primary effects of antibiotics on different members of the community. However, it is not representative of the microenvironment that microbes encounter during infection. Wound fluid represents a much more complex microenvironment (Kadam et al., 2021; Loffler et al., 2013), and various *in vitro* models address this by incorporating serum or simulated wound fluid to mimic wound conditions (Kadam et al., 2021; Said et al., 2014; Slade et al., 2019; Thaarup & Bjarnsholt, 2021). Indeed, previous studies have shown that serum media affects antibiotic tolerance (Blanchard et al., 2014; Kadam et al., 2021; Ledger et al., 2022; Morrison et al., 2020; Young et al., 2023). Thus, further work should ascertain if similar protective and community effects are observed with serum-based media. Future work could also benefit from exploring long-term effects of co-culture, identifying if prolonged growth in mixed communities affects the evolution of resistance. This phenomenon has been demonstrated previously, where co-culture of bacterial species resulted in increased rate of antimicrobial recalcitrance (Beaudoin et al., 2017; Bottery et al., 2021; Elias & Banin, 2012; Quinn et al., 2022; Tognon et al., 2017). Co-culture has also been shown to affect virulence (Baldan et al., 2014; Chan et al., 2018; Tognon et al., 2019), and transcriptional profiling can shed light on the effects of interspecies interactions at the level of gene expression. Another area to explore in the future is the effect of commensals on community dynamics. Oates & McBain (2016) used the phenolic foam (as used in this study) to explore how prior colonisation with commensals affected the integration of pathogens in biofilms. Another study of *S. epidermidis* (commensal) and *S. aureus* (pathogen) found that co-infecting with the two bacteria reduced growth of the pathogen in a 3D epidermal model and inhibited invasion of deep layers of the epidermis by *S. aureus* (Kohda et al., 2021). Thus, in the context of the three-species model developed in the current study, interesting questions arise, such as – does the incorporation of commensals affect population dynamics and survival ratios during antibiotic exposure?

Another key aspect to consider is that various *in vitro* models use a binary approach to studying microbial communities – focusing on planktonic or biofilm forms, as has been done in this study. However, within wound microbial communities, it is likely that infections exhibit both biofilm and planktonic forms of bacteria simultaneously. Lichtenberg et al. (2023) recently showed that chronic wounds indeed harbour a heterogenous population consisting of biofilms, single cells and small clusters of a few bacteria. The use of a simple static culture system with three microorganisms does not accurately mimic the complex and dynamic environment of wound infections. This highlights the need for more advanced multispecies, continuous culture systems that better replicate physiological conditions. For instance, the Constant Depth Film Fermenter (CDFF) can maintain biofilms at a consistent thickness while simulating natural shear forces, making it ideal for studying biofilm structure and treatment responses. Similarly, the Drip Flow Biofilm Reactor (DFR) can replicate the dynamic conditions of intermittent flow and surface exposure to air, providing valuable insights into biofilm behaviour in diverse settings. However, the use of these advanced systems is not always advantageous, especially in the context of this study. These systems can be complex, time-consuming, and expensive to set up and maintain. For studies focused on understanding basic population dynamics and antibiotic interactions, simpler static models can still provide valuable insights while being more accessible and easier to replicate.

Expanding the number of organisms in the model to include a broader range of species would increase its physiological relevance. However, creating and maintaining a complex microbial community presents significant challenges, such as the need for advanced chemicals and reagents, standardising the community composition and ensuring stable growth conditions. This has been explored in other studies. For example, Kucera et al. (2014) developed a four-species biofilm model consisting of *S. aureus, E. faecalis, B. subtilis* and *P. aeruginosa*, requiring culture on Bolton broth base, gelatine, porcine plasma and porcine erythrocytes. Brown et al. (2022) and Townsend et al. (2016) have even developed *in vitro* models consisting of both bacterial and fungal members, thus showcasing the possibility of inter-kingdom mixed community models. However, other studies have highlighted the difficulties associated with maintaining growth of different organisms in co-culture. In an *in vitro* mixed community model consisting of six species, Hill et al. (2010) demonstrated that *P.*

aeruginosa and *S. aureus* dominated, while *S. oralis, B. fragilis, P. anaerobius*, and *M. luteus* were present at very low levels or not detected.

Despite the limitations of this study, it highlights the complex nature of interspecies interactions in mixed communities and its effects on antibiotic susceptibility. While further exploration of the intricacies of these interspecies interactions is outside the scope of this study, performing co-culture experiments with two strains at a time can help further dissect protective and community effects.

E. coli, S. aureus, and *S. pyogenes* represent a novel three-species combination relevant to wound infections, which has not been extensively explored before. This is significant because Gram-negative bacteria like *E. coli* are often overlooked in clinical culture diagnoses, where the focus is typically on identifying the predominant organism considered causative. While identifying causative pathogens is crucial, it is equally important to consider the entire wound microbial community. This study highlights that the presence of an organism with antibiotic resistance can influence the effectiveness of treatment for sensitive organisms, providing a protective effect and potentially complicating the treatment of wound infections. This is even more concerning considering that one of the antibiotics for which this protective effect was seen is flucloxacillin, the first line of treatment for diabetic foot infections. Data from various studies suggest that flucloxacillin prescribing has been increasing in the UK (Fleming et al., 2007; Francis et al., 2016), and various MRSA strains exhibit resistance to flucloxacillin (Nathwani et al., 2010). Thus, understanding how microbial members of mixed community infections behave in the presence of flucloxacillin provides insights into antibiotic resistance and the effects of treatment outcomes.

Antibiotic resistance represents a global health issue. To address this issue, exploratory studies such as this one are crucial in understanding the effects of antibiotics on microbial populations. In light of rising AMR, it is important to view resistance from the perspective of a ‘community’ property. This study showcases that even in the case of antibiotics that are resistant to β-lactamases as well as bacteriostatic antibiotics, there exist protective effects in mixed microbial communities. Thus, there are other factors that influence treatment outcomes, going beyond the antibiotic susceptibility of an individual strain.

3.6 Conclusion

In conclusion, this study has provided valuable insights into the population survival dynamics and interspecies interactions within mixed-species bacterial communities, particularly under antibiotic exposure. The development and optimisation of rapid and reproducible two-species and three-species models for planktonic and biofilm states have demonstrated the intricate and sometimes protective interactions that can occur among different bacterial strains. These findings underscore the complexity of wound infections, where diverse microbial communities and biofilm formation contribute to treatment challenges. By incorporating flucloxacillin and doxycycline, this study provides novel insights into dynamics of mixed-species communities when treated with clinically relevant antibiotics. By quantifying the effects of antibiotics on these mixed communities, this research highlights the importance of considering the polymicrobial nature of infections in clinical management and the potential for certain species to influence the overall resistance profile and treatment outcomes. This showcases why there is a need to view antibiotic resistance as a ‘community’ property. Understanding these population dynamics is crucial for developing more effective therapeutic strategies and improving treatment outcomes in the context of chronic wound infections.

Chapter 4

Effects of Host DNA Depletion on the Wound Microbiome

4.1 Introduction

4.1.1 Human DNA contamination in microbiome studies

The Human Microbiome Project (HMP), initiated in 2007 by the National Institute of Health, sought to map the microbial communities at various body sites in healthy individuals (Creasy et al., 2021). This comprehensive project encompassed diverse sites, including the airways, skin, oral cavity, gastrointestinal tract, and vagina, subjecting extracted DNA samples to 16S rRNA sequencing and whole genome shotgun sequencing. 16S rRNA sequencing has been routinely used to classify bacteria into taxonomic groups for decades, and its first reported use was in the 1980s (Clarridge, 2004; Kolbert & Persing, 1999; Woese, 1987; Woese et al., 1985). The 16S ribosomal RNA (rRNA) gene is a widely used marker for studying bacterial communities due to its presence in all bacteria and its conserved nature across different species. The gene is approximately 1,500 base pairs long and consists of nine hypervariable regions (V1-V9) that provide species-specific signature sequences useful for bacterial identification and phylogeny. The hypervariable regions are flanked by highly conserved sequences, making it possible to design universal primers that amplify the 16S rRNA gene from a wide range of bacterial taxa. In the HMP, the V3-V5 regions of the 16S rRNA gene were predominantly used for amplification. The HMP typically amplified a region of about 450-500 base pairs, which provided a balance between taxonomic resolution and the technical limitations of sequencing technologies available at the time. This approach allowed for the comprehensive characterisation of the human microbiome across various body sites.

While 16S rRNA sequencing has effectively identified bacteria to the family or genus level, it often lacks the taxonomic resolution needed to differentiate closely related species due to the short reads generated by many sequencing technologies (Janda & Abbott, 2007). For instance, species within the *Enterobacteriaceae* family, such as *Escherichia coli* and *Shigella,* which can have 99% similarity in the 16S gene (Halimeh et al., 2021), often cannot be distinguished using this method. Limited taxonomic resolution has been a significant challenge to achieve precise microbial identification in microbiomes. However, the integration of advanced sequencing technologies can enhance the resolution and accuracy of 16S-based microbial community analysis (Caporaso et al., 2011; Matsuo et al., 2021; Nygaard et al., 2020; Szoboszlay et al.,

2023). HMP addressed these limitations of 16S through the adoption of shotgun sequencing and long-read sequencing technologies, offering a broader, untargeted approach that enhances taxonomic resolution. Despite these advancements, the introduction of metagenomic whole genome shotgun sequencing exposed a new challenge - contamination of samples with human DNA. This challenge had not been encountered with the 16S approach since it enabled the amplification of only bacterial DNA, thus automatically removing human DNA from the picture.

Analysis of the whole genome shotgun sequencing data from the HMP has identified variable amounts of human DNA (reads), with samples such as saliva, nasal and vaginal samples found to contain over 90% human reads (Gevers et al., 2012; Marotz et al., 2018). Similarly, high levels of human DNA have been found in metagenomic studies of the wound microbiome, with up to 98% human reads reported (Heravi et al., 2020; Kalan et al., 2019; Verbanic et al., 2022; Verbanic et al., 2019). The human genome is significantly larger than microbial genomes, with a difference of approximately 1000-fold in terms of base pairs. Thus, when human cells are present in microbiome samples, a substantial proportion of the extracted DNA is human DNA. During metagenomic or shotgun sequencing of these samples, most of the sequenced DNA will consist of human DNA fragments. This predominance of human DNA in these samples means that a significant portion of the sequencing effort targets human DNA, overshadowing microbial DNA. This bias can severely impact the precise identification and quantification of microbial communities, particularly those present in low abundance. As a result, the presence of human DNA in microbiome samples can mask the microbial signal. This highlights a significant challenge in human microbiome studies; with all samples collected for studying the human microbiome, there is invariably the presence of both microbial and human cells in different ratios based on the sample type and the sampling technique utilised. In the case of samples with high amounts of human DNA, the sequencing platform's ability to accurately identify microbial species, especially those present in low abundances, is diminished. Pereira-Marques et al. (2019) demonstrated a marked decrease in taxonomic resolution when sequencing samples of a mock bacterial community spiked with increasing amounts of host (mouse) DNA. In this study, for samples with ≥90% mouse DNA, whole metagenomic sequencing using the Illumina platform was unable to detect the lowest abundance species in the mock community (~10^4 16S rRNA copies). Additionally, in

a synthetic sample containing 90% mouse DNA and 10% mock community, reduction of the sequencing depth reduced the ability to detect low abundance species. This highlights the significant impact of host DNA on the sensitivity and accuracy of microbial detection. Hence, there is a need to identify methods of removal of host DNA in microbiome samples.

4.1.2 Methods for removal of host (human) DNA in microbiome samples

To tackle the challenge of human DNA contamination, several strategies have been developed to remove human DNA from microbiome samples. Broadly, these strategies fall into three major categories - pre-sequencing, during sequencing and post-sequencing (Figure 4.1).

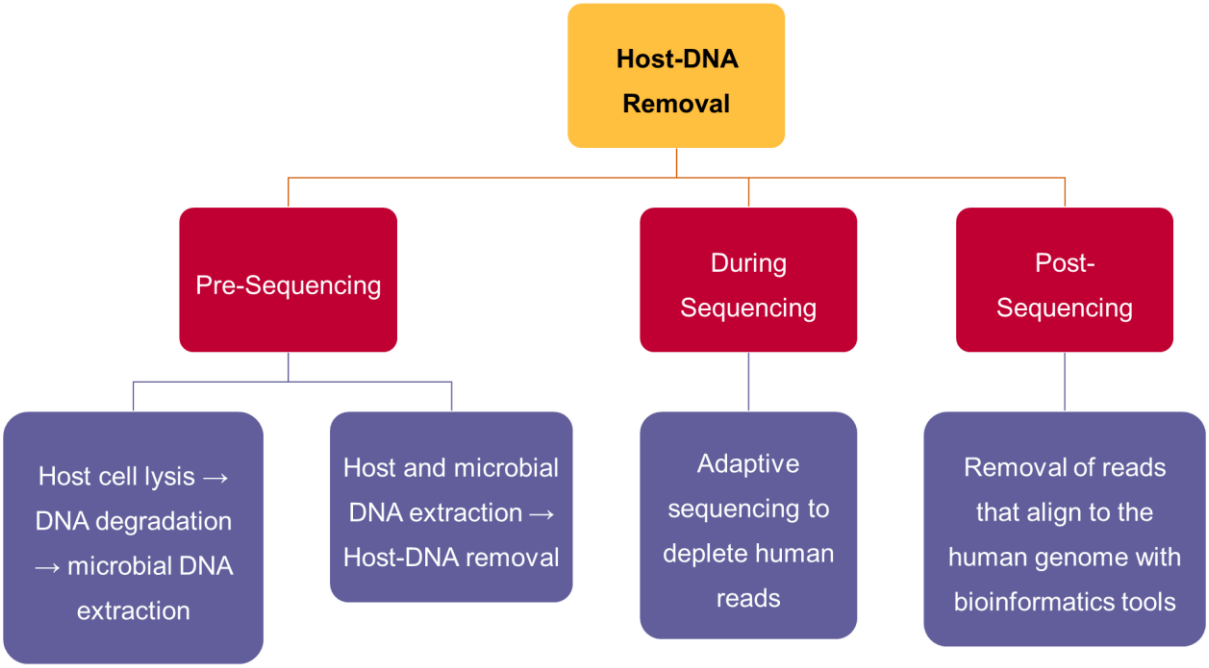

Figure 4.1: Host DNA removal strategies. *Host DNA removal can be performed at different time points with respect to sequencing. Prior to sequencing, host DNA can be removed along with the extraction process. With advances in adaptive sequencing, host DNA can be removed during the sequencing run itself. The most common method of removing host DNA is post sequencing, using numerous bioinformatics tools to remove reads that align to the human genome.*

The pre-sequencing approach involves the depletion of human DNA prior to sequencing, specifically during the DNA extraction stage. This approach can be further divided into methods that eliminate host DNA prior to extraction and those that remove host DNA post-extraction. Currently, there are a range of commercially available kits designed to selectively eliminate human DNA while preserving microbial DNA (HostZERO Microbial DNA Kit; MolYsis; NEBNext Microbiome DNA Enrichment Kit; SPINeasy Host Depletion Microbial DNA Kit). These kits may employ specific binding agents or chemical treatments that target and remove human DNA from the sample. Additionally, detergent-based methods utilising chemicals (like saponin or triton) have been previously described (Bruggeling et al., 2021; Hasan et al., 2016; Nelson et al., 2019; Shi et al., 2022), where human cell lysis is induced by these reagents followed by DNAse treatment to degrade the human DNA released from the lysed cells. There are also post-extraction methods of human DNA removal, such as the NEBNext Microbiome Enrichment Kit (New England Biolabs), which uses protein-bound magnetic beads to bind methylated DNA and remove it from the extracted DNA sample.

Another method to remove host DNA is by applying adaptive sampling principles during sequencing. Adaptive sampling is a method that uses real-time experimental observations to adjust the experiment as it is being run. Oxford Nanopore Technologies applied this approach to sequencing, leading to the advent of ‘adaptive sequencing’ for their platform – a method to enrich or deplete specific DNA molecules (Loose et al., 2016; Martin et al., 2022; Weilguny et al., 2023). Nanopore sequencing involves the use of flow cells with nanopores embedded in a membrane (Wang et al., 2021b). When DNA or RNA molecules pass through these nanopores, a change in the electrical signal across the membrane occurs. This change is detected and used to identify the nucleotide sequence of the molecule. In adaptive sequencing, as a DNA molecule passes through the nanopore, a preliminary segment of its sequence is read in real-time (a few 100 base pairs) (Martin et al., 2022). The platform compares the preliminary sequence against pre-defined criteria or a reference database to determine whether the sequence is of interest. Based on the criteria, an instantaneous decision is made to continue sequencing or to reject the molecule. To deplete host DNA, each DNA molecule is compared to the human genome reference sequence as it passes through the pore, and if matched, the molecule is ‘rejected’ from the pore

and not sequenced further (Marquet et al., 2022). This allows the pore to be accessible to sequence another DNA molecule. This method of eliminating host DNA involves removal at the stage of sequencing. A study of the vaginal microbiome showed that this method of human DNA depletion could reduce human reads to below 5% with minimal changes in the microbiome composition, except for certain low abundance genera (Marquet et al., 2022).

The most commonly used method for host DNA removal is the post-sequencing *in silico* removal of human reads, a technique that was employed in the Human Microbiome Project (Gevers et al., 2012). This approach involves aligning sequenced reads against a human reference genome and removing those that match, a method that is supported by various bioinformatics tools designed for this purpose (Rumbavicius et al., 2023; Schmieder & Edwards, 2011). This approach, however, harbours significant drawbacks, notably the potential misclassification of microbial DNA as human due to sequence similarity, leading to unintended removal. For example, a study showed that the alignment tools of choice for human read removal can influence false positive rates, with these tools misidentifying bacterial reads as human reads (Bush et al., 2020). Additionally, since human DNA reads are filtered out after sequencing, this still reduces the overall data coverage available for microbial DNA.

Nanopore adaptive sequencing, though promising (Hewel et al., 2024; Lin et al., 2023; Marquet et al., 2022; Martin et al., 2022; Wrenn & Drown, 2023), is still in its infancy and has not yet become the standard method for microbiome studies. Currently, most studies continue to rely on *in silico* removal of human reads to focus on microbial DNA. Some studies have explored pre-sequencing host depletion methods, which physically remove human DNA before sequencing. However, the efficiency of these methods can vary significantly depending on the sample type. This variability poses challenges in consistently enriching microbial DNA, highlighting the need for further optimisation and validation of these techniques in diverse sample settings.

4.1.3 Efficiency of pre-sequencing host depletion methods

Previous comparisons of host depletion methodologies have revealed inconsistent results regarding the efficacy of human DNA removal. For example, Marotz et al. (2018) reported that samples treated with the NEBNext Microbiome Enrichment kit exhibited no significant reduction in human reads in comparison to untreated samples within an oral microbiome study. The same study reported that the MolYsis Basic kit, the Qiagen QIAamp DNA Microbiome kit and a method employing osmotic lysis and propidium monoazide led to substantial decreases in human reads, accounting for approximately 62.9%, 29.2% and 8.5% of the control respectively. Furthermore, in a separate study on spiked urine samples, Street et al. (2019) found that the MolYsis Basic kit effectively reduced human DNA to 0.3% of the control. In another study of sputum from individuals with cystic fibrosis (Klosinska et al., 2022), the QIAamp DNA Microbiome kit was found to be most efficient in reducing human DNA. When evaluated on human intestinal biopsies, the NEBNext Microbiome Enrichment kit and QIAamp DNA Microbiome kit were most effective, enriching microbial reads and reducing host DNA (Marchukov et al., 2023). These findings highlight the variability in depletion efficacy across different studies.

Research has also shown that the sample type influences the efficacy of a selected host depletion method. In a study comparing effective host DNA removal across various swab types (vaginal, oral and skin), Ahannach et al. (2021), reported that the HostZERO Microbial DNA kit was superior in reducing human DNA from vaginal and saliva samples in contrast to the QIAamp DNA Microbiome kit. However, for skin swabs, which already have low amounts of human DNA in comparison to vaginal and saliva swabs, no significant effect on human DNA was reported.

Also, host depletion methods can potentially remove any extracellular DNA or free microbial DNA, enabling the study of only intracellular microbial DNA. For instance, Amar et al. (2021) showed that pre-digestion of skin microbiome mock samples using benzonase, which targets all types of DNA, can not only reduce human DNA from 81% to 0.37% but also remove any extracellular or free microbial DNA from dead cells.

In methods utilising detergent-based host depletion, prior studies have explored the effects of varying detergent concentrations to lyse host cells in microbiome samples.

This process includes the enzymatic digestion of DNA released from lysed host cells using DNAse, followed by DNA extraction from remaining intact microbial cells. Using this approach, Charalampous et al. (2019) found that saponin at a 2.2% concentration significantly reduced human DNA in sputum, bronchoalveolar lavage, and endotracheal tube aspirate samples by approximately 10^4-fold, as measured by qPCR. Similarly, the use of saponin was effective in lowering human DNA to 1.1% of a control in spiked urine samples (Street et al., 2019). In contrast, another study of human colon biopsies found that proteinase K treatment, followed by exposure to 0.0125% saponin or 0.006% triton and a DNAse treatment step reduced human DNA while increasing bacterial signal detected via qPCR (Bruggeling et al., 2021). When evaluating the effects of saponin and triton along with DNAse treatment on spiked cerebrospinal fluid and nasopharyngeal aspirate samples, 0.1% and 0.025% saponin and 0.025% triton reduced the percentage of human DNA compared to non-depleted control samples (Hasan et al., 2016). While the authors do report an increase in the pathogen-to-human DNA ratio, indicating enrichment of pathogen DNA, a reduction in the percentage of pathogen DNA compared to the non-depleted sample is also observed. Hence, when considering the efficacy of host-depletion methods on the microbiome, it is important to take into account the effects of these methods on microbial DNA.

4.1.4 Effects of pre-sequencing host depletion methods on bacterial DNA

Although host depletion methods are intended to exclusively eliminate human DNA, the processes of cell lysis and DNAse treatment could inadvertently affect the microbial population or residual microbial DNA. For example, in the study by Ahannach et al. (2021), it was reported that the HostZero Microbial DNA Kit introduced bias against Gram-negative bacteria in skin, saliva, and vaginal samples. This kit operates by lysing eukaryotic cells for host depletion. However, such lysis-based methods can also impact microorganisms, specifically Gram-negative bacteria, which possess a thinner outer cell layer than Gram-positive bacteria. The study by Charalampous et al. (2019) utilised a saponin-based host depletion method for respiratory samples, and while other respiratory pathogens remained unaffected, a 5.7-fold reduction was reported for *S. pneumoniae* following host depletion. Hence, although host depletion methods are often effective at reducing human reads, their impact on microbial DNA

warrants careful consideration. Given this potential risk, it is crucial to evaluate host depletion methods not just for their ability to reduce host DNA but also to identify their impact on bacterial DNA.

4.1.5 Host depletion in the wound microbiome

Currently, only one previous study has evaluated different host depletion methods in the context of the wound microbiome - a study in 2020 explored the QIAamp DNA Microbiome Kit and HostZERO microbial DNA Kit on infected diabetic foot infection tissue biopsies (Heravi et al., 2020). The team reported that both kits significantly reduced human DNA contamination, resulting in a ten-fold increase in bacterial DNA percentage compared to a control method using 16S rRNA sequencing. Whilst the use of 16S rRNA Illumina sequencing has various limitations, including difficulty in differentiating between closely related bacterial species and copy number variation (Větrovský & Baldrian, 2013), the findings of this study highlight the impact of host DNA even in targeted 16S molecular sequencing.

The disparate outcomes observed across studies employing commercial kits and detergent/DNAse-based methods for different sample types underscores the need to evaluate host DNA depletion protocols for sample types relevant to the study. In addition, some commercial kits are more suited to handling liquid samples (such as blood, saliva) or biopsies, which can be homogenised; however, very few commercial kits have manufacturer-provided protocols for swab samples. This further underlines the importance of optimising a host depletion method suited to the specific sample type of interest.

Another crucial factor to consider when evaluating different host depletion techniques is the method of quantification. Although relatively few in number, previous studies have explored various commercial kits as well as chemical agents to deplete host DNA in human oral, skin, vaginal, respiratory and urine samples, as well as from diabetic foot infection biopsies and colon biopsies, yet the methods of evaluation and quantification methods in these studies differ. For example, in a study that used the NEB Microbiome Enrichment Kit on frozen lung tissue samples 16S copy number was used to indicate microbial enrichment and human DNA copy number was not reported

(Wiscovitch-Russo et al., 2022). In yet another study, 16S and 18S copy numbers were estimated using qPCR and the ratio of 18S/16S copy number was used as a proxy for human DNA reduction (Heravi et al., 2020). Many studies have also used qPCR to evaluate the effects of host depletion methods, while others have used Illumina-based metagenomic sequencing or advanced sequencing technologies such as Nanopore sequencing to understand the microbial profiles of host-depleted samples (Amar et al., 2021; Charalampous et al., 2019; Hasan et al., 2016; Nelson et al., 2019; Street et al., 2019).

Selecting suitable sequencing technology is a critical consideration in microbiome research. Eliminating human DNA enhances the sequencing of microbial reads, but it must be paired with the proper sequencing technology for optimal results. In this study, Nanopore MinION sequencing by Oxford Nanopore Technologies was employed, which facilitates rapid, long-read sequencing and greater/enhanced taxonomic resolution.

4.1.6 Nanopore sequencing

Nanopore sequencing offers real-time, long-read sequencing capabilities that have revolutionised genomics research (Oxford Nanopore Technologies). This technology uses nanopores - tiny pores in membranes - through which DNA or RNA molecules pass, enabling the detection of nucleotide sequences based on changes in ionic current (Lu et al., 2016). An electrical potential is applied across the membrane, creating an ionic current that flows through the nanopore. As the DNA/RNA molecule passes through each nanopore, it partially blocks the flow of ions, causing a disruption in the ionic current. Each type of nucleotide affects the current differently due to their unique sizes and shapes and the sequencing device measures these changes in ionic current in real time to interpret the nucleotide sequence.

Oxford Nanopore provides several platforms tailored to different research needs. The Flongle is designed for smaller-scale experiments, offering a cost-effective solution for rapid, smaller sequencing runs. The MinION, a portable and versatile device, supports more extensive sequencing projects with the capability to generate up to 48 Gb of data in a single run, making it ideal for fieldwork and clinical applications. For more

extensive, high-throughput requirements, the GridION integrates five MinION flow cells, allowing parallel sequencing and substantial data output, which is suitable for complex projects needing large-scale genomic data. The MinION flow cell provides higher throughput with the ability to generate significantly more data per run, up to 48 Gb per MinION flow cell in comparison to 2.8 Gb per Flongle flow cell (Oxford Nanopore Technologies).

A key benefit of this sequencing platform is its capacity for generating long reads, ranging from 500 base pairs (bps) to tens of thousands of base pairs in length, with the longest reported read being >4 Mb (Oxford Nanopore Technologies). These long read lengths, combined with its ability to produce real-time data, portability, and provisions of PCR-free library preparation, render MinION sequencing a powerful tool for a wide range of applications, including microbial genomics, metagenomics, human genetics, cancer research, environmental monitoring, and microbiome studies (Baker, 2022; Ciuffreda et al., 2021; de Vries et al., 2022; Glinos et al., 2022; Jain et al., 2018).

The platform's ability to identify a broad spectrum of genera and uncover microbiome diversity, augmented by continuing improvements to the platform, has the potential to deepen our understanding of microbiomes significantly (Ciuffreda et al., 2021; Kerkhof, 2021; van der Loos et al., 2021; van der Helm et al., 2017). Consequently, MinION sequencing has emerged as a popular choice for microbiome studies in recent years. Nevertheless, there are some limitations to this sequencing approach, including the relatively high error rate compared to other sequencing methods, the need for specialised software and data analysis tools, and the relatively high cost of the MinION device and associated consumables (Kerkhof, 2021). Some studies have reported high error rates previously (up to 20% for certain species) (Chen et al., 2021; Delahaye & Nicolas, 2021); this is highly influenced by the basecaller used (Wick et al., 2019).

Ongoing advancements in kit and flow cell chemistries, coupled with improvements to basecalling, are addressing these challenges. For example, the use of Guppy's high-accuracy basecalling mode and updated versions of the basecaller have demonstrated reduced error rates (Delahaye & Nicolas, 2021).

Given the limited studies on the effects of host DNA depletion on the wound microbiome, this study aimed to evaluate various host depletion methods on biological

samples relevant to the skin and wounds. Combining this approach with advanced sequencing technologies such as Oxford MinION sequencing will deepen our understanding of the potential impact of host depletion on the wound microbiome. This study will shed light on whether host depletion can improve our understanding of the wound microbiome and determine the most effective protocol for skin and wound swab samples.

4.2 Aim and Objectives

The aim of this chapter was to evaluate various host depletion strategies to understand the impact of host DNA depletion on wound microbiome samples.

The objectives were as follows:

- Identify appropriate host DNA depletion from existing literature for application to skin/wound microbiome swab samples.
- Establish a methodology for comparing different host DNA depletion protocols against a standard non-depletion DNA extraction protocol.
- Evaluate the impact of these protocols on skin samples containing a known quantity of Gram-positive and Gram-negative bacterial species.
- Implement the refined protocols for wound swabs to examine the effects of various methods.
- Characterise the wound microbiome profile in the samples using an appropriate bioinformatics pipeline.

4.3 Materials and Methods

4.3.1 Swab sample collection

4.3.1.1 Skin swabs

To test the effects of different host depletion and DNA extraction methods on human DNA, skin swabs from a healthy volunteer were collected using regular flocked swabs (VWR, 300-0224). Pre-moistened skin swabs were obtained from the posterior auricular regions of either ear. All swabs were stored at -80°C upon collection. Before extraction, the skin swabs were spiked with known quantities of wound-relevant pathogens.

4.3.1.2 Wound swabs

To understand the effects of host DNA depletion on human DNA and the wound microbiome, wound swabs from amputated limbs were collected from Castle Hill Hospital (REC19/NE/0150). Six swabs were collected from each wound to allow comparison between different methods. All swabs were stored at -80°C upon collection.

4.3.2 Spiking skin swabs with bacteria

To look at the effects of the DNA extraction and host depletion methods on specific Gram-positive and Gram-negative bacteria, skin swabs were spiked with known quantities of *Staphylococcus aureus* (MS6) and *Pseudomonas aeruginosa* (ATCC 28753). All bacterial cultures were grown as described before (see Chapter 2).

Swab heads were cut and placed into a 1.5 mL tube in sterile conditions. In separate tubes, bacterial cultures were adjusted to the appropriate CFU/mL and combined in a 1:1 ratio to achieve 10^7 of each species in the mixture. This mixture was centrifuged (4000xg) to obtain a bacterial pellet. Bacterial pellets were resuspended in 400 µL of 1X sterile PBS. Swab heads were added to the bacterial suspension for 10 minutes to allow the head to be fully saturated. Tubes containing the swab head were then briefly

vortexed to release the contents of the swab head into the PBS and bacterial suspension. Finally, the swab head was removed using sterile forceps and pressed against the tube walls to collect the liquid it retained. The swab head was discarded, and the resultant eluent was put forward for DNA extraction and host depletion. Each spiked sample was divided into two aliquots; one aliquot underwent extraction using the PowerSoil Pro Kit (serving as the control), and the other was treated using a host-depleted method. This approach ensured that each host-depleted sample had a corresponding 'matched' paired sample which had not undergone host DNA depletion. This facilitated the direct comparison of the two samples, enabling quantification of changes in both human DNA and spiked bacterial DNA levels.

4.3.3 Wound swabs: pooling samples

Prior to the extraction of DNA from wound swabs, each swab head was cut and placed into a 1.5 mL tube containing 300 µL of 1X sterile PBS. Tubes were then vortexed to allow the contents of the swab head to be released into the PBS. Finally, swab heads were removed using sterile forceps and pressed against the tube walls to collect the liquid they retained. The swab heads were then discarded. The six resuspensions in PBS were then pooled together to create one 'Pooled Sample' constituting one biological replicate. The pooled sample was then divided into equal parts for DNA extraction. This was done for three independent wounds, resulting in three biological replicates. DNA was extracted from these pooled samples using the methods detailed below.

4.3.4 DNA extraction and host depletion methods

To understand the effects of each host depletion method, each matched (or pooled) sample was extracted by a control method which did not deplete any host DNA. The DNeasy PowerSoil Pro Kit (Qiagen, 47014) served as the 'control' method for this purpose. This study explored five different methods of host depletion methods outlined in Table 4.1.

Table 4.1: Host depletion and DNA extraction methods

Host Depletion Method	**DNA Extraction Method**	**Host depletion method details**	**Sample Type Tested**
MolYsis Complete 5 Kit	Part of the kit	Host cell lysis → DNAse treatment → DNA extraction of microbial cells using Proteinase K	Spiked Skin Swabs Wound Swabs
NEBNext Microbiome Enrichment Kit	PowerSoil Pro Kit	DNA extraction → removal of methylated (host) DNA using magnetic beads	Spiked Skin Swabs Wound Swabs
Proteinase K, Saponin and DNAse based	PowerSoil Pro Kit	Proteinase K-based digestion → Host cell lysis → DNAse treatment → DNA extraction of microbial cells	Spiked Skin Swabs Wound Swabs
5% Saponin and DNAse based	PowerSoil Pro Kit	Host cell lysis → DNAse treatment → DNA extraction of microbial cells	Spiked Skin Swabs Wound Swabs
0.0125 % Saponin and DNAse based	PowerSoil Pro Kit	Host cell lysis → DNAse treatment → DNA extraction of microbial cells	Wound Swabs alone

Two commercial kits, MolYsis Complete 5 and NEBNext Microbiome Enrichment Kit, were evaluated for their efficiency in deleting host DNA. Previous studies have also evaluated the ability of saponin and DNAse treatment to deplete host DNA. Given the diversity of methods applied to various sample types, two methods were adapted for swab samples. The first method involved proteinase K, a low saponin concentration and DNAse-based DNA degradation. The second method employed saponin only, at a higher concentration than the first method and DNAse-based DNA degradation.

After preliminary experiments with these methods with axenic cultures of bacteria (see results section), a second saponin-based method was also added to host depletion methods. This method was similar to the Proteinase K, Saponin and DNAse-based

method, except the Proteinase K step was removed (0.0125% Saponin and DNAse-based).

For each host depletion method, three biological replicates were processed, and DNA extraction using the PowerSoil Pro served as the control. Extracted DNA was quantified using the Qubit 3.0 Fluorometer (Invitrogen, Q33216) and the Qubit High Sensitivity (HS) Assay Kit (Invitrogen, Q32854).

4.3.4.1 DNeasy PowerSoil Pro Kit

The DNeasy PowerSoil Pro Kit (Qiagen, 47014), which employs bead-beating and chemical lysis of cells, was used based on the manufacturer's recommendations. The aliquot for extraction via this method was mixed with 800 µL of the PowerSoil Pro Kit lysis buffer and added to the bead tube provided. Lysis was performed using a vortex tube adapter for 5-10 minutes at the maximum speed of the vortex. After lysis, DNA extraction was performed as recommended by the manufacturer. The final DNA was eluted from the spin column in 50 µL of the elution buffer. DNA was quantified and stored at -20°C till further required.

4.3.4.2 MolYsis Complete 5 Kit

The MolYsis Complete 5 kit (VHBio, 05D321050) is a microbial DNA isolation kit that lyses host/animal cells with a chaotropic buffer, degrades DNA released from these lysed cells using a DNAse, degrades microbial cell walls, removes PCR inhibitors and then purifies the microbial DNA on spin columns. DNA was extracted using the recommended protocol provided by the manufacturer. The aliquot obtained after spiking skin swabs or after pooling wound swabs was added to a 2 mL sterile tube, and Buffer SU was used to make up the volume to 1 mL as recommended by the manufacturer. The DNA was finally eluted in 70-100 µL of deionised water. DNA was quantified and stored at -20°C till further required.

4.3.4.3 NEBNext Microbiome Enrichment Kit

The NEBNext Microbiome Enrichment Kit (New England BioLabs, E2612S) removes host DNA by capturing it on protein-bound magnetic beads based on CpG methylation. Before the use of this kit, DNA from swabs was extracted using the DNeasy PowerSoil Pro Kit. 500 ng of the DNA obtained (or 50 ng if a low DNA quantity was obtained) was used for further host depletion by the NEBNext Microbiome Enrichment Kit by following the manufacturer's recommended protocol. DNA was purified using Ampure XP Beads as recommended. The DNA was air-dried and resuspended in 20 µL of Buffer CD6 (elution buffer from the DNeasy PowerSoil Pro Kit). DNA was quantified and stored at -20°C till further required.

4.3.4.4 Proteinase K, 0.0125% Saponin and DNAse-based host DNA depletion

This method was based on previously published work (Bruggeling et al., 2021), and carried out using modifications. Sterile 1X PBS was added to the sample to make up the volume to 297 µL. The sample was then treated with 3 µL of ~20 mg/mL Proteinase K (Thermo Scientific, EO0491) for 15 minutes on a thermomixer at 56°C and 1000 rpm. The sample was centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatant was discarded, and the pellet was resuspended in 88 µL of 0.0125% sterile saponin, 10 µL buffer 10× Turbo DNAse buffer (Invitrogen AM2238) and 2 µL TurboDNAse (2 Units/µL). After a short vortex, the sample was then incubated for 30 minutes on a thermomixer at 37°C and 1000 rpm to allow for host cell lysis and degradation of released DNA. After 30 minutes, 1 mL PBS was added to the sample and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was then removed, and the pellet was resuspended in 800 µL of Buffer CD1 (the lysis buffer of DNeasy PowerSoil Pro Kit). DNA extraction was immediately performed using the DNeasy PowerSoil Pro Kit method.

4.3.4.5 5% Saponin and DNAse-based host DNA depletion

The 5% saponin method was modified from a previously published study (Charalampous et al., 2019). 1X sterile PBS was added to make up the volume of the sample to 250 µL. To this, 200 µL of 5% saponin was added, and the sample was briefly vortexed and incubated at room temperature for 10 minutes. 350 µL of nuclease-free water was added to the sample and further incubated at room temperature for 30 seconds. The sample was centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatant was discarded, and the pellet was resuspended in 88 µL of 1X PBS, 10 µL buffer 10× Turbo DNAse buffer (Invitrogen AM2238) and 2 µL TurboDNAse (2 Units/µL). This was incubated for 30 minutes on a thermomixer at 37°C and 1000 rpm to allow for degradation of DNA released from lysed cells. After 30 minutes, 1 mL PBS was added to the sample and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was then removed, and the pellet was resuspended in 800 µL of Buffer CD1 (the lysis buffer of DNeasy PowerSoil Pro Kit). DNA extraction was immediately performed using the DNeasy PowerSoil Pro Kit.

4.3.4.6 0.0125% Saponin and DNAse-based host DNA depletion

The 0.0125% saponin method was based on previously published work (Bruggeling et al., 2021), and carried out using modifications. Samples were centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatant was discarded, and the pellet was resuspended in 88 µL of 0.0125% sterile saponin, 10 µL buffer 10× Turbo DNAse buffer (Invitrogen AM2238) and 2 µL TurboDNAse (2 Units/µL). After a short vortex, the sample was then incubated for 30 minutes on a thermomixer at 37°C and 1000 rpm to allow for host cell lysis and degradation of released DNA. After 30 minutes, 1 mL PBS was added to the sample and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was then removed, and the pellet was resuspended in 800 µL of Buffer CD1 (the lysis buffer of DNeasy PowerSoil Pro Kit). DNA extraction was immediately performed using the DNeasy PowerSoil Pro Kit.

4.3.5 DNA storage

All samples were stored in 1.5 mL LoBind® Tubes (Eppendorf) at -20°C.

4.3.6 DNA quantification

DNA quantification was performed using Qubit fluorometer 3.0 (ThermoFisher Scientific) and Qubit dsDNA HS (high sensitivity) Assay Kit as recommended by the manufacturer. Briefly, 10 µL of the provided DNA standard was added to 190 µL of the Qubit working solution, and both standards were used to calibrate the Qubit fluorometer before quantifying any samples. For all samples, 1 µL of DNA was added to 199 µL of the Qubit working solution to quantify DNA.

4.3.7 Quantification of host and bacterial DNA using qPCR

To quantify the amount of host DNA and the spiked bacterial DNA extracted from spiked skin swab samples, SYBR Green-based quantitative real-time PCR (qPCR) was used. qPCR primers validated and used in previous studies that were specific to human DNA or either of the spiked bacterial species were chosen (Table 4.2). All primers were obtained in lyophilised form (Eurofins) and resuspended in molecular grade nuclease-free water (Fisher Bioreagents BP2819-100) to obtain a concentration of 100µM. Primers were further diluted to 10µM (working solution) and stored at -20°C till use.

Table 4.2: qPCR primers

Species	Primer Sequence	Amplicon Length (bps)	Annealing Temp	Target gene	Reference
Homo sapiens	FP: *AGGCCTGCTGAAAAT GACTG*	115	60°C	*kras*	Bruggeling et al. (2021)

Species	Primer Sequence	Amplicon Length (bps)	Annealing Temp	Target gene	Reference
	RP: *TGGATCATATTCGTC CACAAAA*				
Staphylococcus aureus	FP: *GTTGCTTAGTGTTAA CTTTAGTTGTA* RP: *AATGTCGCAGGTTCT TTATGTAATTT*	154	53°C	*nuc*	Kilic et al. (2010)
Pseudomonas aeruginosa	FP: *AGCGTTCGTCCTGCA CAAGT* RP: *TCATCCTTCGCCTCC CTG*	145	55°C	*ecfX*	Mangiaterra et al. (2018)

A 20 µL reaction mixture consisting of 10 µL 2X qPCRBio SyGreen Blue (PCR Biosystems, PB20.16-05), 400nM each of forward and reverse primers, 2 µL template DNA and nuclease-free water (Fisher Bioreagents BP2819-100) was prepared. All qPCR reactions were prepared in a dedicated PCR laminar flow hood under sterile conditions. The Applied Biosystem Step-One Plus instrument was used for all qPCRs with the following PCR cycling conditions: an initial temperature of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C (or the appropriate annealing temperature for each primer pair) for 1 minute. A melt curve was also performed for each qPCR experiment, with an initial temperature of 95°C for 15 seconds, followed by 60°C for 1 minute, and increments of 0.3°C up to 95°C, and a final incubation at 95°C for 15 seconds.

A known amount of genomic DNA (gDNA) from each species (*Homo sapiens*, *Staphylococcus aureus, Pseudomonas aeruginosa*) (Table 4.3) was used to produce

standard curves for each experiment with the respective primer pair. All gDNA standards were prepared in nuclease-free water (Fisher Bioreagents BP2819-100) using a 1:10 dilution between subsequent standards. Based on the amount of DNA in nanograms, all gDNA standards were quantified as copy numbers using the formula:

$$\boldsymbol{Number\ of\ copies} = \frac{Amount\ of\ DNA\ (ng) \times 6.0221\ \times 10^{23}\ molecules/mole}{\left(Length\ of\ DNA\ (bps) \times 660\frac{g}{mole}\right) \times 1 \times 10^{9}\ ng/g}$$

Table 4.3: Range of genomic standards used for qPCR

Species	**Number of gDNA standards**	**gDNA copy number range in standards**	**Dilution factor between subsequent standards**
Homo sapiens	5	10-10^4	1:10
Staphylococcus aureus	7	10-10^7	1:10
Pseudomonas aeruginosa	7	10-10^7	1:10

The standard curve produced was used to calculate copy number per swab of each spiked sample. This was used to assess the efficiency of different host depletion methods and evaluate their effects on bacterial DNA. A two-sample t-test was performed using OriginPro 2021b (version 9.8.5.204) to test the statistical significance of human DNA quantified in each host-depleted method compared to the control PowerSoil method.

4.3.8 Nanopore MinION sequencing of DNA from wound swabs

4.3.8.1 DNA cleanup

Prior to sequencing, magnetic bead-based DNA cleanup using Ampure XP beads (Beckman) was performed on all extracted DNA (except NEB Microbiome Enrichment Kit extracted samples, as the protocol already contained a cleanup with Ampure XP beads). Ampure XP beads were added to each DNA sample at 1.8 times the sample volume and mixed gently via pipetting until a homogenous mixture was obtained. The samples were then incubated at room temperature for 5 minutes. The sample tubes were placed on a magnetic rack to collect the beads and left till a clear solution was visible. After the beads had separated from the solution, leaving the tube on the rack, the clear liquid was removed and discarded. The tubes were kept on the magnetic stand, and the beads were washed twice with 200 µL of 80% ethanol. Tubes were allowed to air dry to ensure all the ethanol had evaporated. DNA was eluted in 20 µL of Buffer CD6 (elution buffer from the DNeasy PowerSoil Pro Kit). DNA was quantified and stored at -20°C till further required.

4.3.8.2 Library preparation and sequencing

Nanopore sequencing was performed in-house (n=2) or in collaboration with the Genomics Services Laboratory at the University of York (n=1). The Rapid PCR Barcoding Kit (SQK-RPB004) was used for library preparation according to the manufacturer's recommendations with minor modifications. Fragmentation was performed at 30°C for 1 minute and at 80°C for 3 minutes. PCR cycles were increased to 15 during the barcoding process. The library was prepared by pooling equimolar quantities of barcoded samples and adaptor ligation was performed. The library was loaded onto a MinION FLO-MIN109 R9.4.1 flowcell for sequencing. Raw sequencing data (fast5 files) was obtained for further analysis.

4.3.9 Bioinformatics analysis

4.3.9.1 Basecalling

Basecalling was performed using Guppy Basecalling Software, Oxford Nanopore Technologies plc. version 6.4.2. Fastq files were used for further analysis.

4.3.9.2 Filtering, quality control and classification to analyse microbiome data

To perform filtering and QC and to make taxonomic assignments, a Snakemake workflow developed at the University of Hull was used (Freiheit, 2023). Filtering was performed within the workflow using a minimum length requirement of 100 bps, and a Phred quality score of 7. Taxonomic classification was performed using Kraken2, using the default parameters in the workflow (threshold score of 0.05 and minimum score parameter of 10). The workflow also converted the classified reads from a kraken file to a biom file. This biom file was used for further analysis.

4.3.10 Data analysis and statistics

The biom file obtained from the workflow was imported using the *phyloseq* package in RStudio (version 2.4.1). All downstream analyses to quantify bacterial and eukaryotic reads, visualise abundance at different taxonomic levels and find unique species were carried out using microbiome-specific R packages available. Statistical analysis was performed using the *rstatix* package in R. Normal distribution of the data was confirmed using the Shapiro-Wilk Normality Test. Next, a one-way ANOVA was performed, followed by a Tukey post-hoc test to identify significantly different groups. This statistical analysis was performed for both bacterial and eukaryotic read groups. For non-parametric data, a Kruskal-Wallis test was performed to test for statistical significance between groups.

4.4 Results

This study investigated various host DNA depletion prior to sequencing and compared it to a non-depleted method (Figure 4.2). The Qiagen DNeasy PowerSoil Pro Kit (formerly known as the MoBIO PowerSoil Kit), a kit widely used in human microbiome research and recommended by the Human Microbiome Project was selected for this purpose. Two commercial kits, MolYsis Complete 5 Kit and NEB Microbiome Enrichment Kit), were employed for host DNA depletion. In addition, two different protocols adapted from the literature involving saponin for host-cell lysis followed by DNA degradation using DNAse treatment were examined.

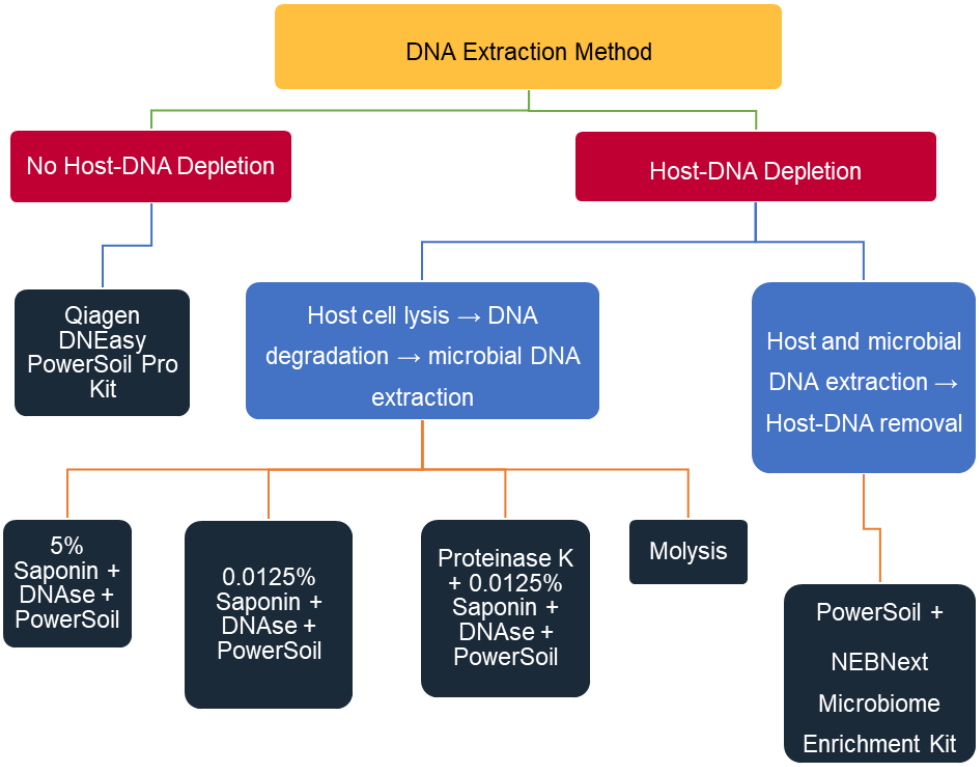

Figure 4.2: Overview of host DNA depletion and DNA extraction methods used in this study.

4.4.1 Host DNA depletion reduces human DNA in skin swab samples

To evaluate the ability of different host depletion methods, preliminary optimisation and assessment assays were conducted on skin swabs from a healthy individual from the posterior auricular regions of either ear spiked with two common wound pathogens (Gram-negative and Gram-positive organisms).

Utilising qPCR and employing standard curves with species-specific primers targeting *P. aeruginosa*, *S. aureus,* and *H. sapiens* genomic DNA (see Chapter 8), accurate measures of the DNA quantities of each species present in spiked samples were achieved. Comparisons were made between the amount of DNA obtained through host-depleted methods and the PowerSoil Pro Kit (control), with results expressed as a percentage of the latter. All four methods tested exhibited a significant reduction in human DNA content (Figure 4.3) ($p<0.0001$). Specifically, the 5% Saponin + DNAse and Proteinase K + 0.0125% Saponin + DNAse methods successfully decreased human DNA to less than 1% when compared to the PowerSoil Pro Kit alone.

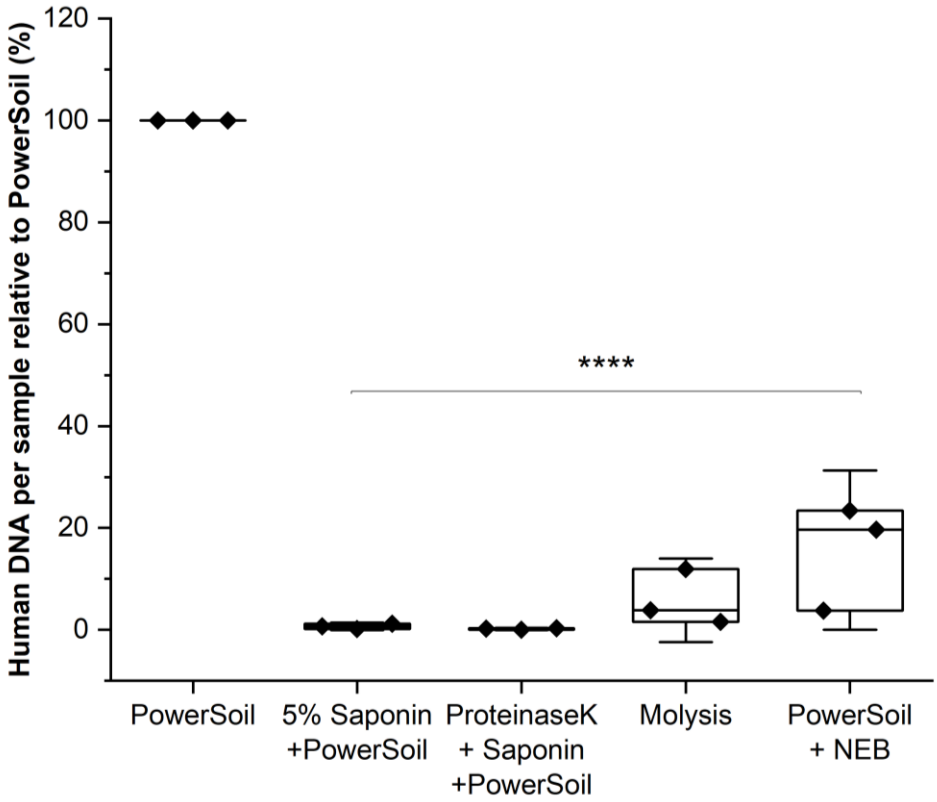

Figure 4.3: Percentage of human DNA per sample isolated from spiked skin swabs with different extraction methods. *Number of human DNA copies in PowerSoil extracted samples were considered as 100% and human DNA copy numbers in all other DNA extraction methods were expressed as a percentage of this (N=3). p<0.0001*

4.4.2 Host DNA depletion affects spiked bacterial DNA in skin swabs

To assess the impact of host DNA depletion methods on bacterial DNA, known quantities of two key wound pathogens (*P. aeruginosa* and *S. aureus*) were used. Samples that were subjected to host depletion via 5% Saponin + DNAse exhibited a spiked bacterial profile which most closely resembled that of the control (PowerSoil) (Figure 4.4). Proteinase K- and MolYsis-based methods had a clear reduction effect on the amount of *P. aeruginosa* DNA detected ($p<0.01$ and $p<0.05$ respectively). The 5% saponin-based method also reduced *P. aeruginosa* DNA quantified, though this was not statistically significant. With the exception of MolYsis, host depletion methods improved DNA quantities extracted from *S. aureus*, though this was not statistically significant.

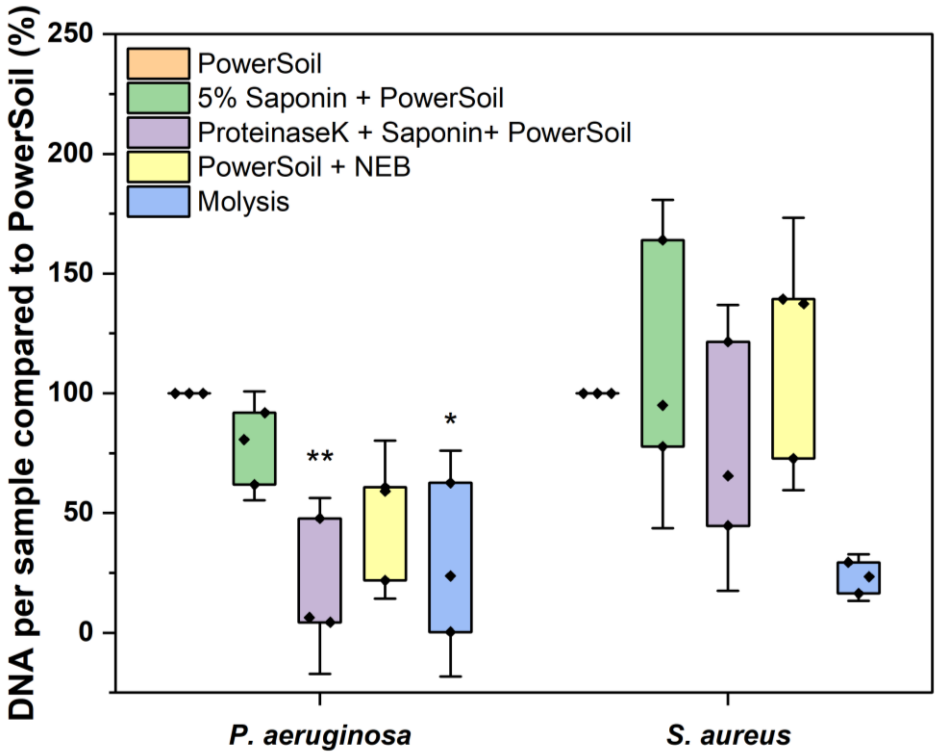

Figure 4.4: Percentage of P. aeruginosa and S. aureus DNA per sample isolated from spiked skin swabs with different extraction methods. *Number of DNA copies in PowerSoil extracted samples were considered as 100% for each bacterial species and DNA copy numbers in all other DNA extraction methods were expressed as a percent of this (* $p<0.05$, ** $p<0.01$).*

4.4.3 Host DNA depletion decreases human reads and improves bacterial read depth in wound microbiome sequencing

The impact of the host depletion methods on the wound microbiome and their efficacy in removing human DNA from wound swabs was evaluated using wound swab samples. Pilot data identified a potential impact of Proteinase K on microbial communities; therefore an additional host depletion method was included without Proteinase K. MolYsis depleted samples were excluded from analysis due to limited amplification during the PCR barcoding stage. As such, the host depletion methods evaluated for wound samples were as follows: PowerSoil, 5% Saponin, 0.0125% Saponin, Proteinase K, NEBNext.

Wound swabs that underwent extraction without host depletion (PowerSoil method) predominantly consisted of human reads (94.6%), with bacterial reads being minimal (5.4%) (Figure 4.5). Different host depletion methods were seen to influence the amount of human vs bacterial reads to variable extents. The 5% Saponin + DNAse-based host depletion method demonstrated the most substantial reduction in human reads ($p<0.0001$), decreasing from 94.6% to an average of 0.6% (Figure 4.5). This reduction was accompanied by a notable increase in bacterial reads, rising from 5.4% in PowerSoil to 99.4% in the 5% Saponin + DNAse method. Both Proteinase K + 0.0125% Saponin + DNAse and 0.0125% Saponin + DNAse methods did exhibit a reduction in human reads (23.4% and 18.9% respectively) and an accompanying increase in bacterial reads (76.6% and 81.1% respectively). However, the extent of these changes was not as pronounced as that observed with the 5% Saponin method. The NEB Microbiome Enrichment Kit did not significantly affect the human or bacterial reads.

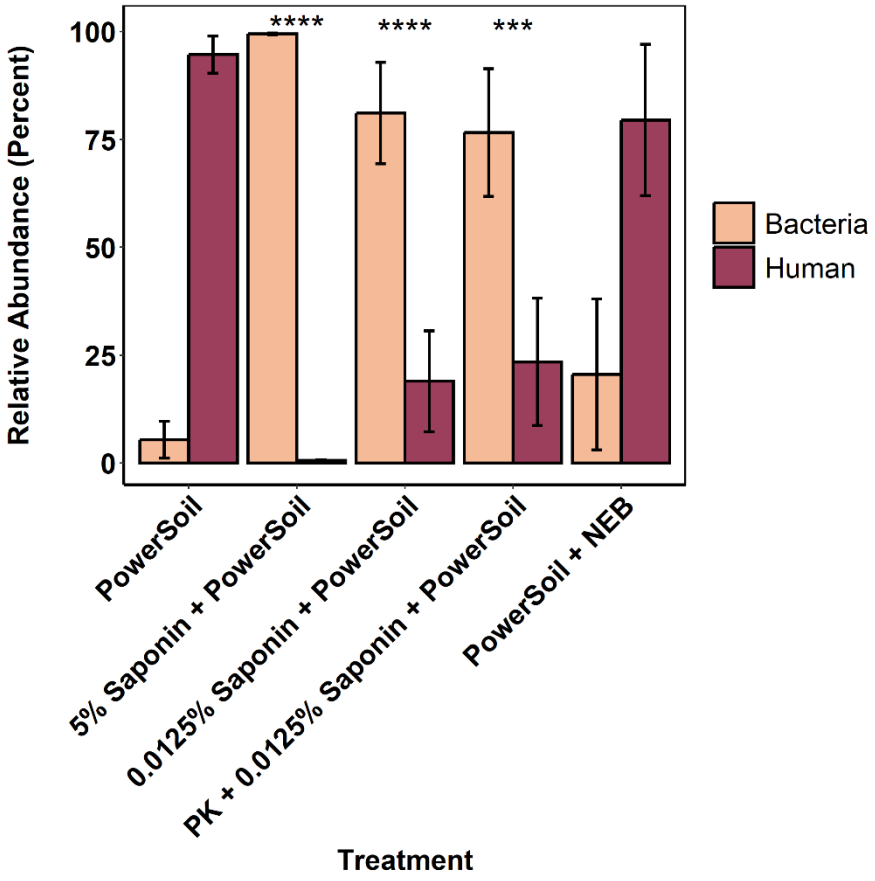

Figure 4.5: Relative abundance of Human vs Bacterial reads from wound swabs extracted with different host-depletion methods *(Mean±SD, N=3, *** p<0.001, **** p<0.0001).*

4.4.4 Host DNA depletion improves detection of more unique species

Each host depletion method was compared to the respective control (PowerSoil) to identify unique species. This analysis allowed us to determine the number of bacterial species exclusively detected in each host-depleted method and absent in the respective PowerSoil-extracted sample (and vice versa). All three saponin-based methods consistently detected more unique species than PowerSoil (Figure 4.6). The unique species detected included known wound inhabitants such as *S. epidermidis, S. agalactiae* and *S. dysgalactiae*. There was variability between the biological replicates of each method; however, this is expected due to the inherent diversity of microbial communities in different wounds.

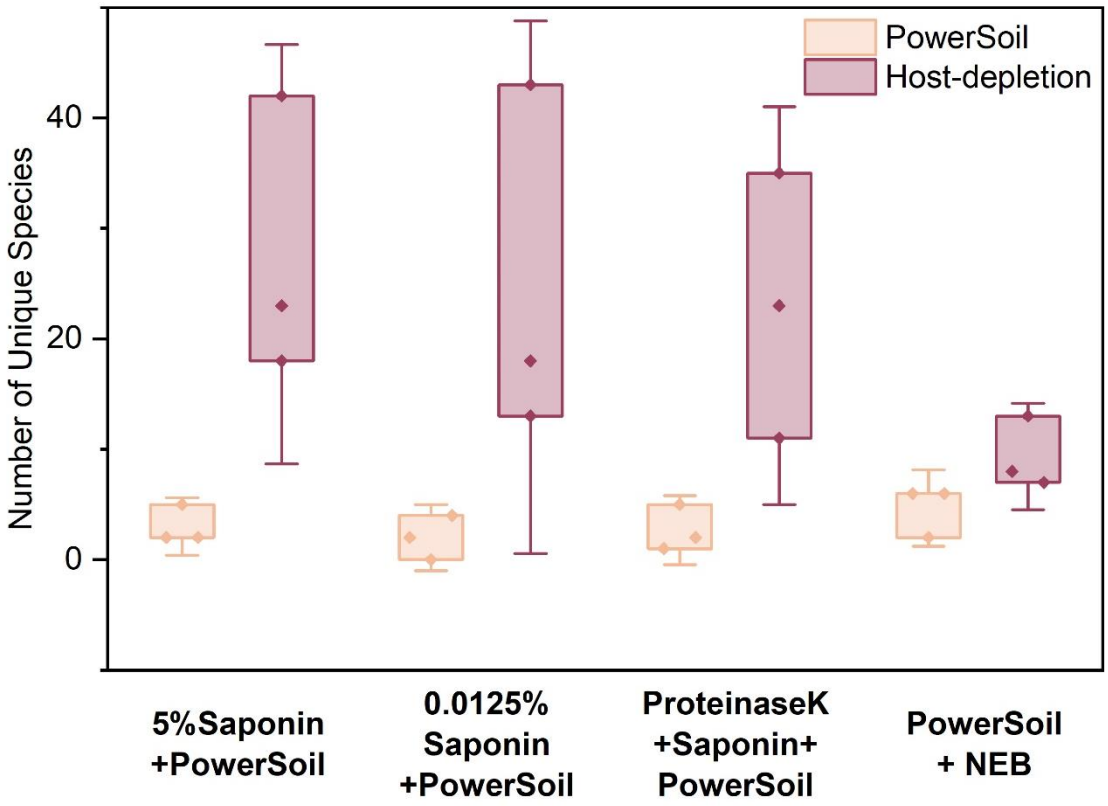

Figure 4.6: Number of unique species in the wound microbiome. *The number of unique species detected in each host-depleted method were compared to PowerSoil method (Mean±SD, N=3).*

4.4.5 Host DNA depletion enriches common bacterial taxa

While saponin-based methods enabled the detection of unique bacterial taxa, their effects on the abundance of common bacterial taxa were also investigated. To achieve this, comparisons between each host depletion method sample and its corresponding PowerSoil control were made in order to identify bacterial species detected by both methods. The ratio of the abundance of one common taxon in the host-depleted method to its abundance in the PowerSoil method was calculated as an indicator of enrichment. This process, repeated for every commonly detected taxon and across all biological replicates, provided a distribution of log-enrichment values for all common taxa (Figure 4.7).

All saponin-based methods enriched many common taxa compared to PowerSoil. Very few bacterial species had a lower abundance in the saponin-based depletion methods than PowerSoil (data points in red in Figure 4.7). The high levels of enrichment observed for these saponin-based methods (Figure 4.7) also correlate with the overall increase in bacterial reads that were detected by these methods (Figure 4.5). The NEB kit had the least enrichment of all host-depleted methods tested and, in fact, had multiple species for which the abundance was lowered in the PowerSoil + NEB extracted sample compared to PowerSoil alone.

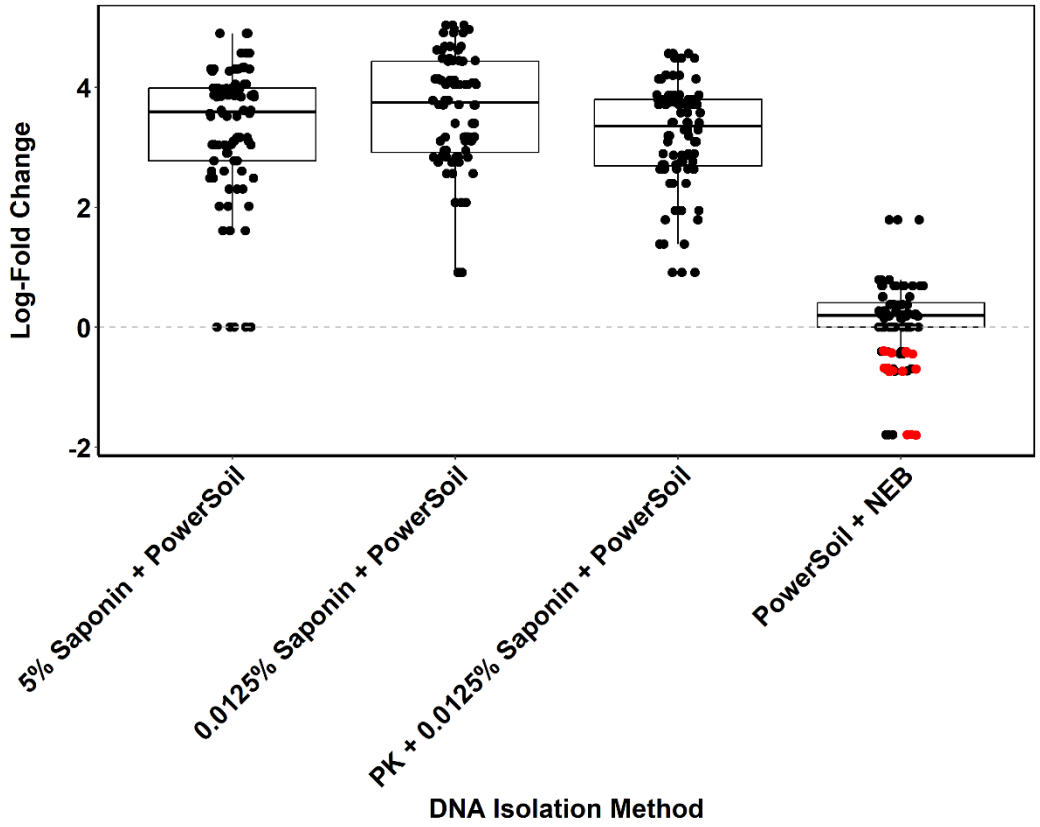

Figure 4.7: Log-Fold Change for common taxa between each host depletion and control method *(N=3). The dotted line corresponds to a log-fold change of 0, indicating no change in abundance of the taxa between the host-depleted method and the control method. Each data point corresponds to the fold change of one single taxon. Red data points indicate taxa for which log-fold change <0 indicating a reduction in abundance in the host-depleted method for that taxon.*

4.4.6 Host DNA depletion preserves phyla-level relative distribution, but increases absolute abundance of various genera

At the phyla level, host DNA depletion did not change the overall profile of each sample when considering the relative abundance (Figure 4.8). When comparing different methods, it is important to not only look at an overview of the microbial profile as indicated by relative abundance but to also look at absolute abundance values which are more indicative of changes in reads for different genera. Indeed, a higher number of reads were observed for numerous genera in the different biological samples tested for host-depleted methods (Figure 4.9). Wound-relevant pathogens such as *Pseudomonas, Streptococcus, Corynebacterium, and Klebsiella* were among the genera with increased abundance in saponin-based methods.

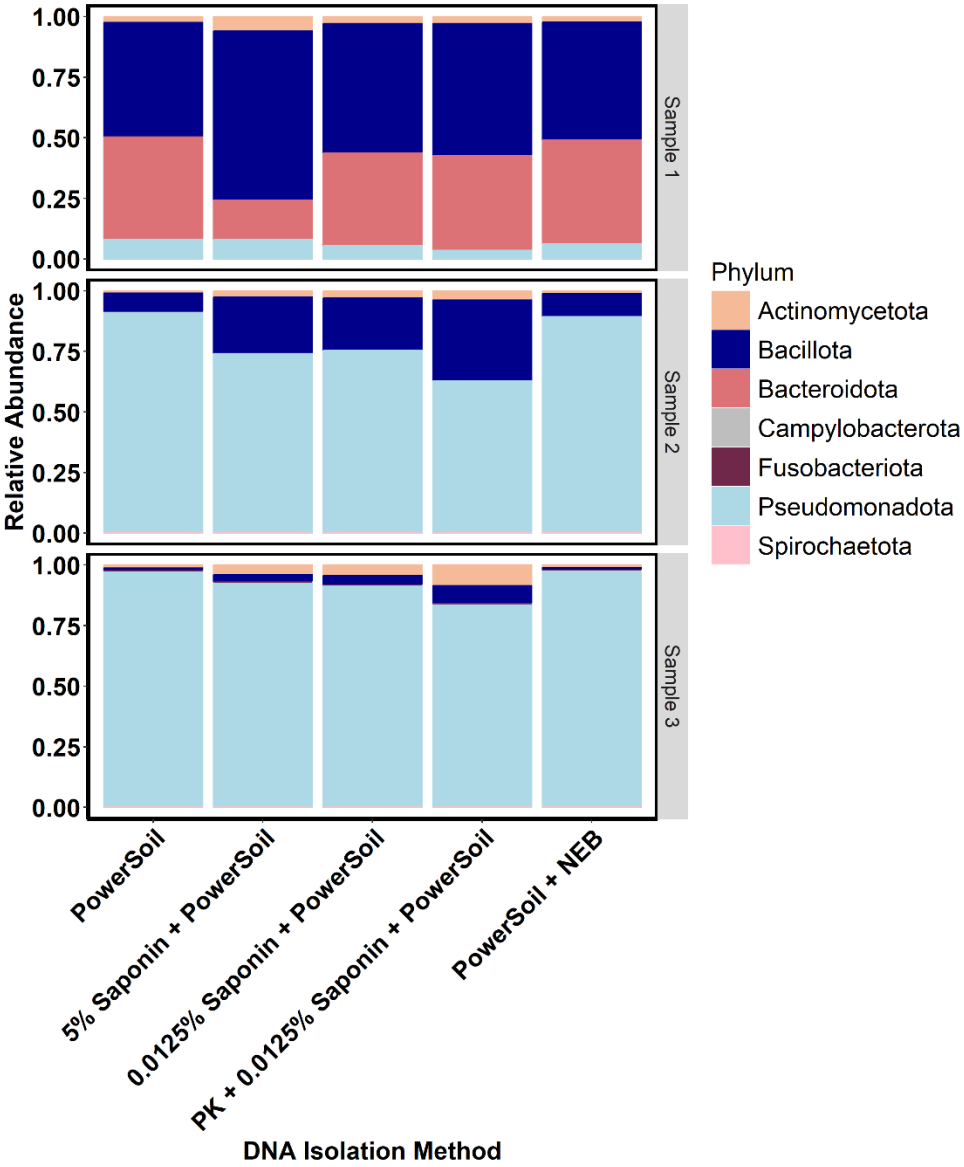

Figure 4.8: Relative abundance of bacterial phyla (3 biological replicates). *DNA was isolated from diabetic wounds using different host depletion methods. The figure represents the distribution of bacterial phyla in each of the three biological replicates (Sample 1, 2 and 3).*

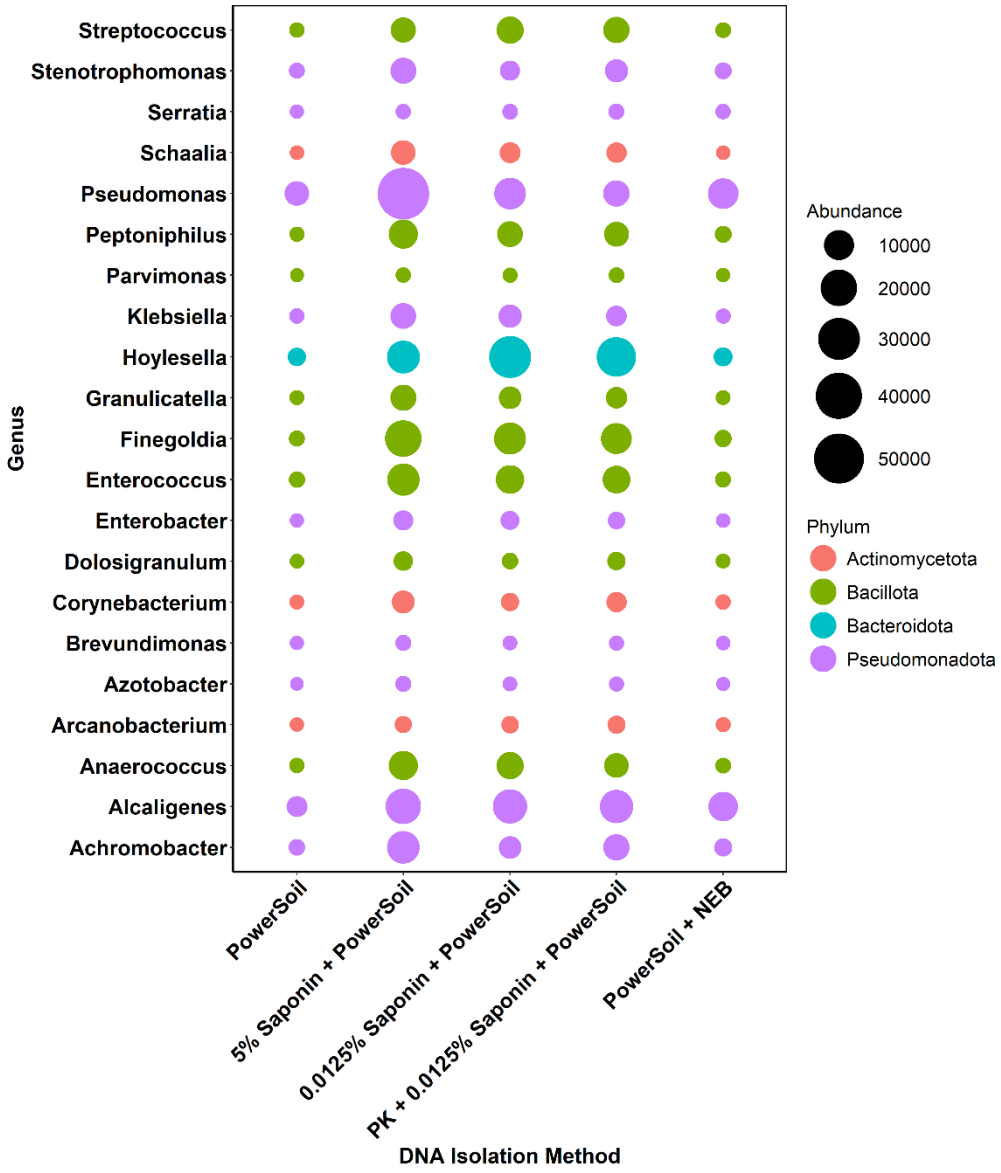

Figure 4.9: Bubble plot indicative of absolute abundance of bacterial genera *isolated from one of the three biological replicates of wound swabs extracted using different host depletion methods. This data shows only the top 10 genera from each replicate.*

4.4.7 Host DNA depletion increases the bacterial alpha diversity profile of wound swabs

Host depletion using saponin-based methods also exhibited increased alpha diversity (Figure 4.10). While the alpha diversity had high variability within a method, this is expected across different biological samples. Despite this variability, the alpha diversity for saponin-based methods was higher than the PowerSoil extraction

method, indicating higher species richness for these methods in comparison to the non-depleted sample.

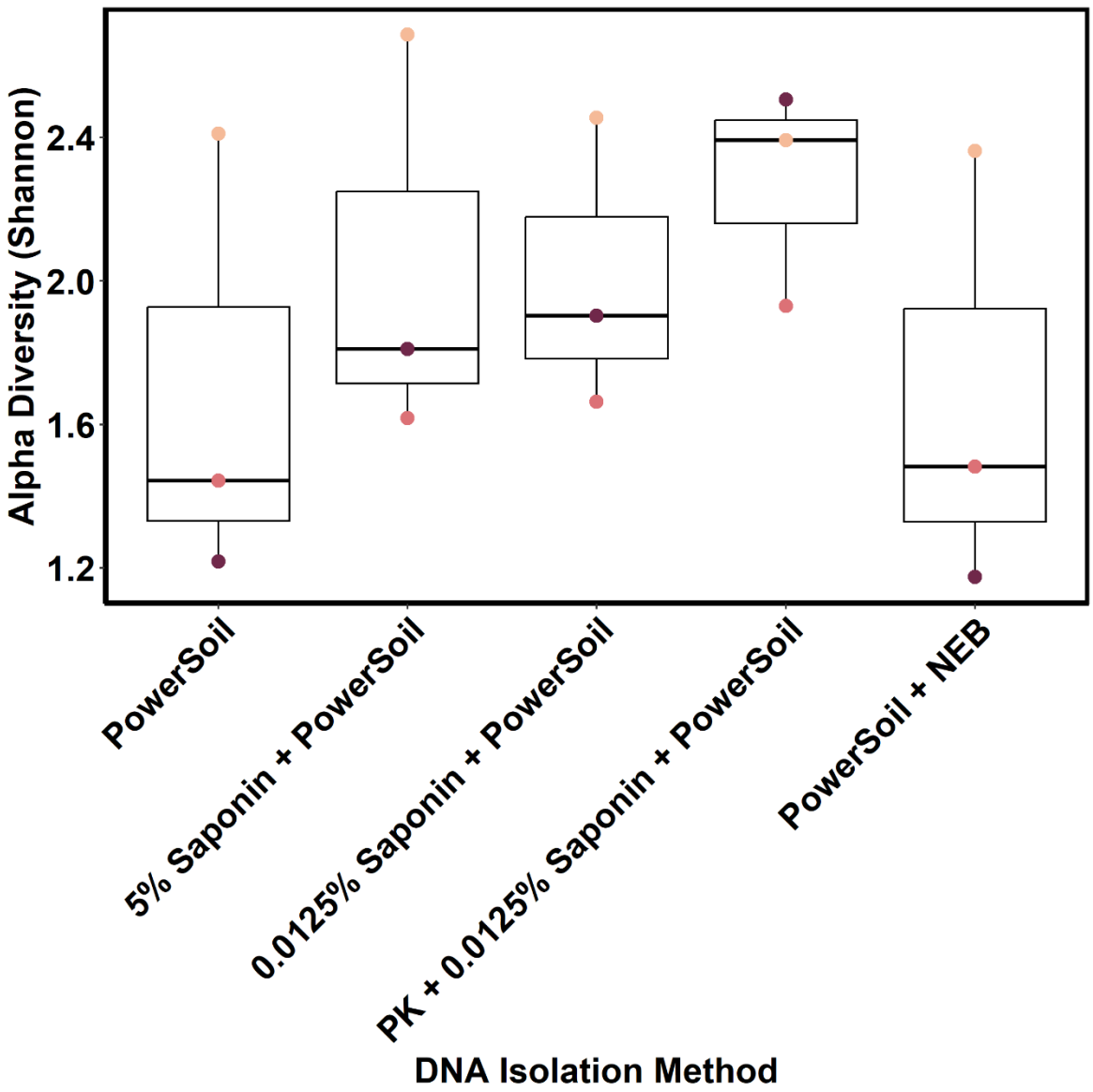

Figure 4.10: Bacterial alpha diversity (Shannon) of wound microbiomes for different host depletion methods and PowerSoil. *The coloured data points indicate the three biological replicates.*

4.4.8 Saponin-based host DNA depletion does not indicate a bias towards Gram-positive bacteria

Since the skin swab pilot study found a reduction in *P. aeruginosa* levels detected via qPCR, the distribution of Gram-negative and Gram-positive bacteria was evaluated in the wound microbial profile (Figure 4.11). The distribution of the two groups of bacteria was different across the three biological replicates, indicating the diverse nature of wound microbiomes. The first wound that was sampled had a more even distribution of Gram-positive and Gram-negative bacteria, whereas the other two wounds were dominated by Gram-negative bacteria. No significant differences were seen in the distribution of Gram-negative bacteria in the different treatment groups ($p = 0.98$, Kruskal-Wallis test), contrasting to the skin pilot study which saw a reduction in *P. aeruginosa* levels.

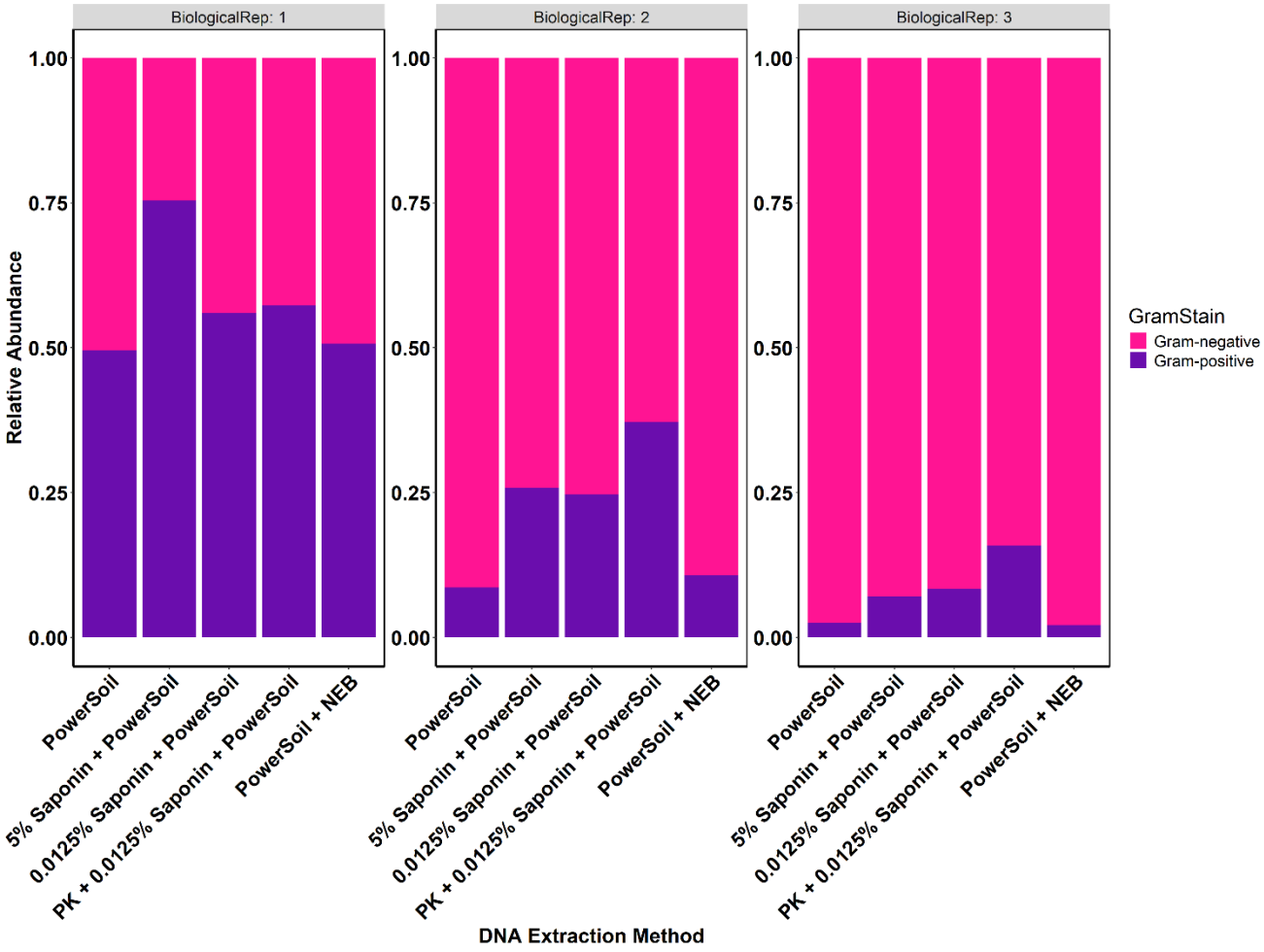

Figure 4.11: Distribution of Gram-negative and Gram-positive bacteria *in the three wound replicates across different host depletion and DNA extraction methods.*

4.5 Discussion

In this study, the goal was to evaluate the effects of host DNA depletion on the wound microbiome, a crucial step in determining the most effective host depletion/DNA extraction method for clinical samples. While this analysis is essential for the completion of this PhD project, it also holds significant value for advancing research into the wound microbiome beyond the confines of this study. Skin was selected as the sampling site for initial comparisons of DNA extraction and host depletion methods due to its accessibility from healthy individuals and the resemblance of its microbial community to a wound's microbial community. It is acknowledged that skin swabs differ from wound swabs in their biological content, specifically the lack of cell and tissue debris, wound fluid, and other factors of the wound microenvironment. Nevertheless, the purpose of using spiked skin swabs was to conduct preliminary investigations to ascertain whether host depletion methods can effectively deplete host DNA and if they affect two major wound pathogens representing Gram-positive and Gram-negative bacteria.

Using spiked skin swabs and qPCR, it was observed that the host depletion methods led to a reduction in human DNA copy numbers, with the largest decrease seen in saponin-based methods. However, the host depletion methods also influenced microbial DNA. Specifically, a reduction in *P. aeruginosa* DNA copy number was noted for the Proteinase K, MolYsis and NEBNext methods, indicating an effect of the host depletion process on Gram-negative bacteria. This is likely due to the impact of Proteinase K and the chaotropic agent in the MolYsis kit on the thin outer membrane of Gram-negative bacteria. Loonen et al. (2013) reported a similar observation, with the MolYsis Complete 5 kit affecting detection rates of Gram-negative bacteria more than Gram-positive bacteria. Lazarevic et al. (2022) demonstrated that the Molzym Ultra-Deep Microbiome Prep kit using proteinase K-based tissue digestion leads to the depletion of Gram-negative bacterial DNA and an overestimation of Gram-positive bacteria.

Interestingly, within this study, with 5% saponin, an increase in the *S. aureus* DNA copy number was seen, possibly due to improved lysis of the thick Gram-positive cell wall. The 5% saponin method had the least effect on Gram-negative *P. aeruginosa*, though a slight reduction was noted. Whilst the four host depletion methods herein

were aimed to remove only human DNA, quantities of spiked bacterial DNA were affected. Additionally, despite using known quantities of bacteria to spike to swabs, a large variability was seen in the bacterial DNA quantified in host-depleted methods. This may be attributed to various possible reasons. Firstly, each skin swab might not absorb the bacterial suspension uniformly, leading to differences in the initial number of bacterial cells. Further, removing the swab head from the suspension might cause some loss of bacterial cells. The use of replicates in the pilot skin swab approach showcases the possible high variability when using host depletion methods. This suggested the need to further test these on wound infection samples and assess the effect on the whole microbial profile.

The initial evaluation using skin swabs underscored the necessity of incorporating a 'matched' control to accurately assess the effects of host depletion methods. Consequently, for the wound swabs, six individual samples were collected from the same wound and pooled to form a single biological replicate. This approach provides an advantage to this study, enabling a more precise evaluation of various host depletion techniques and ensuring that the control and experimental conditions are directly comparable. Additionally, pilot data from skin swabs were instrumental in assessing the impact of different host depletion methods on bacterial DNA. For instance, the pilot study demonstrated that the combination of Proteinase K and 0.0125% saponin reduced *P. aeruginosa* DNA levels. Based on these findings, the methodology for evaluating host depletion in wound swabs was expanded to include an additional approach: the use of 0.0125% saponin without Proteinase K. This addition aimed to further refine the optimisation of host depletion methods in the context of the wound microbiome.

Despite the effectiveness of the MolYsis Kit in reducing human DNA in spiked skin swabs, challenges arose when processing the samples for Nanopore sequencing, where samples extracted by this method failed to amplify during the PCR barcoding procedure. Secondary bead cleanup procedures were undertaken to eliminate any remaining impurities or PCR inhibitors; however, this issue was consistently encountered across multiple biological replicates. As a result, samples extracted via the MolYsis method were excluded from further Nanopore sequencing. While the MolYsis kit has been successfully tested in previous studies of blood, saliva, urine,

cerebrospinal fluid and nasopharyngeal aspirate, amongst others, to deplete host DNA, none of these studies have performed Nanopore sequencing (Hasan et al., 2016; Marotz et al., 2018; McCann & Jordan, 2014; Street et al., 2019; Thoendel et al., 2016). In this study, the DNA obtained from this kit proved unsuitable for Nanopore sequencing.

A significant strength of this study is its focus on evaluating host depletion methods specifically using wound swabs. This approach underscores the necessity of optimising host depletion methods to the specific sample type. Nanopore sequencing data for saponin-based methods demonstrated a reduction in human DNA reads, with a corresponding increase in bacterial DNA reads. Notably, the highest increase in bacterial reads was observed with a 5% saponin concentration. While skin swabs did not show significant differences in host depletion efficiency between the 5% saponin and Proteinase K-based methods, wound samples exhibited a marked disparity. Specifically, the 5% saponin method reduced human DNA reads to less than 1%, whereas the Proteinase K method resulted in a reduction to approximately 23%. The difference in the performance of the 5% saponin and proteinase K methods in wound swabs compared to skin swabs could also be due to the higher levels of host-derived material present in wound swabs.

This study also observed considerable variability in the microbial profiles among the limited number of biological replicates, reflecting the complex nature of the wound microbiome. Each wound sample can differ significantly due to various factors, including the amount of cell debris, wound fluid, and tissue present. This variability not only affects the microbial community but also influences the amount of host DNA present in each sample. Consequently, such differences can impact the efficiency of host depletion methods. Previous studies have similarly documented variability in the effectiveness of these methods (Marotz et al., 2018; Rajar et al., 2022), underscoring the challenges associated with achieving consistent effects across diverse wound samples.

This study assessed the performance of a commercial kit, the NEBNext Microbiome Enrichment Kit, in depleting host DNA from samples. The evaluation, conducted using spiked skin swabs and qPCR, demonstrated the kit's effectiveness in reducing human DNA copy numbers in these samples. However, it also reduced *P. aeruginosa* DNA

quantified. Its performance was also notably diminished in wound swabs, which contain higher levels of human material such as cells, debris, and wound fluid. This reduced efficacy aligns with findings from Marotz et al. (2018), who reported similar challenges with host DNA depletion in saliva samples, which also have a high human DNA content. Additionally, Heravi et al. (2020), observed that, although NEBNext-treated diabetic foot infection tissue samples had a lower 18S/16S ratio compared to non-depleted samples, the ratio remained significantly higher than those achieved with other depletion methods tested in their study. This study corroborates these observations, revealing only a modest reduction in human DNA reads in the wound microbiome (~79% human reads compared to the PowerSoil method), which was not statistically significant ($p = 0.53$). These results suggest that the NEBNext Microbiome Enrichment Kit may not be as effective as other methods for depleting human DNA from samples with high human content.

In fact, both commercial kits evaluated—NEBNext Microbiome Enrichment Kit and MolYsis Complete 5 Kit—were the weakest performing methods of host depletion in this study. This suggests that commercial kits, despite being designed for microbiome enrichment, may not be effective across all sample types. It also underscores the necessity of optimising host depletion methods specifically for the sample type being studied, rather than relying on general-purpose commercial kits. This is particularly important for sampling techniques that use swabs, as most commercial kits do not include specific instructions for swab usage. For example, the MolYsis Complete 5 Kit is designed for liquid samples. While other kits, such as the SPINeasy Host Depletion Microbial DNA Kit, claim compatibility with swab samples, the manufacturer guidelines do not provide detailed instructions for swab use, only specifying a required volume of liquid sample. Although this study used aliquots from spiked/pooled samples and did not directly apply swabs with the kits, it is important to consider the applicability and feasibility of these methods for clinical samples.

In contrast to the commercial kits evaluated in this study, all three saponin-based host depletion methods not only decreased human reads but also enabled the detection of more bacterial species compared to the non-depleted control. Despite observing high variability in the number of unique species detected across different biological replicates, this can be attributed to the complex nature of the wound microbiome. The

combination of sensitive technologies like Nanopore sequencing and diverse microbial communities can significantly influence the effects of host depletion and DNA extraction methods, leading to increased variability. Nevertheless, it was evident that saponin-based host depletion methods revealed more unique species that were undetected by the PowerSoil method. Additionally, these methods increased the absolute abundance of bacterial reads across various genera. Contrary to previous studies that indicated a reducing effect on Gram-negative bacteria (Lazarevic et al., 2022; Loonen et al., 2013), no clear bias towards Gram-positive bacteria was observed with the detergent and enzyme-based host depletion methods used in this study. It is important to note that this is in contrast to the pilot skin swab study, where a reduction in *P. aeruginosa* was observed for the methods tested. This could be attributed to the differences in the sample types, the quantification method (single gene in a pathogen vs the whole metagenomic sample) and the techniques used (qPCR vs Nanopore sequencing). When considering the distribution of Gram-positive and Gram-negative bacteria in the wound microbiome, it is crucial to understand that relative abundance does not provide the complete picture. While relative abundance offers an overview of the microbiome profile, it is inherently a comparative measure. An increase in the abundance of one species will result in a 'relative' decrease of another species, which does not necessarily indicate an actual reduction in the latter's abundance. Therefore, alongside the microbiome relative abundance profile, it is crucial to also consider the overall increased bacterial abundance observed in host-depleted methods.

Three saponin-based host depletion methods were assessed for their effectiveness on wound samples: 5% saponin, 0.0125% saponin, and a combination of proteinase K treatment followed by 0.0125% saponin. All methods successfully reduced human DNA reads and increased the proportion of bacterial reads in the wound microbiome, however, the 5% saponin method proved to be the most effective. This is supported by a previous study where a 5% saponin-based method demonstrated the ability to remove 99.9% of host DNA in respiratory samples, as assessed by qPCR (Charalampous et al., 2019). In another study of endotracheal aspirates, the use of 5% saponin-based depletion was seen to increase microbial reads after Nanopore sequencing, from <1% in the non-depleted samples to ~48% in the host-depleted samples (Yang et al., 2019).

It is important to acknowledge that though the 5% saponin method detected more unique species than the non-depleted sample in the current study, this was comparable across the three saponin-based methods. It is also important to highlight that most of the species that were detected as unique had low abundance, which could indicate that non-depleted samples cannot capture the low abundance species when looking at complex microbial communities. Additionally, the non-depleted samples did have a small number of unique species too (2-5 species), indicating some information loss with the use of 5% saponin-based depletion. Despite this, the overall gain in information on the microbial profiles of the wound microbiome was greater, suggesting that 5% saponin-based host depletion enhanced the understanding of the microbial community. Consequently, the 5% saponin-based method was selected for use in clinical samples to investigate the dynamics of the wound microbiome (see Chapter 5).

This study systematically evaluated multiple host depletion methods using both skin swabs and wound swabs. This step-by-step approach ensured that the most effective method was chosen for wound swabs. By using skin swabs spiked with known amounts of *S. aureus* and *P. aeruginosa*, the study could precisely measure the effectiveness of each host depletion method. This controlled setup provided a clear understanding of how each method impacted not only human DNA levels but also the DNA of Gram-positive and Gram-negative bacteria. Additionally, the use of 'matched' samples for the pilot skin swab work ensured that each host-depleted sample had a direct comparable non-depleted sample. This design allowed for direct comparisons and provided a clear baseline to evaluate the effectiveness of each host depletion method. When assessing the efficacy of host depletion methods on the wound microbiome, wound swabs were pooled. By obtaining multiple swabs from a single wound, pooling them, and then splitting them into aliquots, the study minimised sample variability and allowed for direct comparison, ensuring consistency in the biological sample that was evaluated by different methods. Another key strength of this study is the use of advanced long-read Nanopore sequencing, which has not been previously used to evaluate and compare pre-sequencing host depletion methods, especially in the context of the wound microbiome. Additionally, this study not only evaluates the effectiveness of methods by comparing human and bacterial reads but also delves

deeper into the microbial profile, looking at factors such as unique species and enrichment of common species.

While there are various strengths to this study, its limitations are also acknowledged. The study was conducted with a limited number of biological replicates (samples taken from three different wounds). While this provides valuable preliminary data, more extensive studies would be needed to improve generalisability. Specifically, the samples were taken from chronic wounds which had led to an amputation. However, wound infections are highly complex, and individual variations in cell debris, wound fluid, and tissue components can affect the efficiency of host depletion methods. It would be interesting to see how these host depletion methods perform using samples of varying chronic states and infection grades. Another limitation is the differences in the quantification method used – qPCR for skin swabs compared to Nanopore sequencing for wound swabs. In qPCR, a region of a single gene is used to quantify DNA copies, whereas Nanopore sequencing involves entire DNA fragments throughout the genome. However, despite these differences in quantification approaches, the overall trend observed was similar for skin and wound swabs. Nanopore MinION sequencing, while advanced, can have its own biases and limitations in terms of read accuracy and error rates. The use of a PCR-based amplification for sequencing can also introduce biases.

Additionally, while this study only focuses on pre-sequencing host depletion, recent advances in adaptive sequencing make it a promising approach to host depletion. Adaptive sequencing has been used to selectively deplete the human genome and allowed for microbial enrichment in recent studies (Hewel et al., 2024; Marquet et al., 2022). Gan et al. (2021) demonstrated that combining the HostZero Microbial DNA kit with Nanopore adaptive sequencing significantly enriches microbial reads, with the highest enrichment achieved through the combination of both methods rather than each method alone in the case of respiratory samples. Thus, there is potential to combine pre-sequencing host depletion methods with adaptive sequencing to provide an improved understanding of complex microbial communities.

Despite the limitations of this study, the step-by-step evaluation, starting from spiked skin swabs to actual wound swabs, ensures a methodical approach. Including matched control samples for direct comparison and using replicates enhance the

robustness of the data. Evaluating multiple metrics (human reads, bacterial reads, alpha diversity, number of unique species, and enrichment levels) provides a well-rounded assessment of each method's effectiveness, rather than focusing on just reduction in human reads. Effective host depletion methods enable a more accurate and comprehensive profile of microbial communities by reducing the dominating presence of host DNA. When applied to the exploration of the wound microbiome in the clinical context, the findings of this study have the potential to detect low-abundance microbial species, providing a clearer picture of the wound microbial landscape. This is supported by the fact that saponin-based methods in this study led to the identification of more unique species (ranging from 11 to 43 species) compared to the non-depleted samples, many of which were low-abundance species. The increased presence of bacterial reads in the saponin-depleted samples also indicates a potential for improved detection of not just microbial species but also antibiotic resistance genes, an aspect crucial for understanding the prevalence of antimicrobial resistance within complex microbial communities.

This work also has implications beyond just the area of wound microbiome research. The study underscores the necessity of optimising host depletion methods for different sample types, highlighting the need for tailored approaches in microbiome studies. The systematic approach and detailed methodology presented in the study contribute to the standardisation of host depletion techniques. This can provide a structured approach for the evaluation of host depletion methods relevant to other sample types. The principles and methods demonstrated here can be adapted for studying various environments where host DNA is a major component, such as oral, respiratory, gastrointestinal, and urogenital microbiomes. There is also potential for application to clinical metagenomics, where host depletion approaches can improve detection of pathogens and identification of resistance genes (Charalampous et al., 2019; Miller et al., 2019; Ruppe et al., 2017; Schmidt et al., 2017; Yang et al., 2019).

4.6 Conclusions

In this study, the effects of various host depletion methods on the wound microbiome were systematically evaluated. Initial assessments using qPCR and DNA extracted from spiked skin swabs confirmed that all tested host depletion methods successfully reduced the amount of human DNA. Among these methods, the combination of 5% saponin and DNAse treatment reduced human reads the most in wound samples. This reduction was also accompanied by an increase in the absolute abundance of numerous bacterial species, highlighting the efficacy of host depletion. Notably, the 5% saponin method not only enhanced bacterial reads but also improved the detection of a greater number of bacterial species compared to the PowerSoil (non-depleted) method. Therefore, within the sample types tested, the 5% saponin-based host depletion method emerged as the optimal approach for wound swabs, providing a more comprehensive and accurate understanding of the wound microbiome. This study underscores the importance of refining host depletion techniques to enhance microbiome research, particularly in the context of specific sample types.

Chapter 5

Metagenomic Profiling of Microbial Communities and Antimicrobial Resistance in Diabetic Foot Ulcers: A Longitudinal Approach

5.1 Introduction

Wound healing is a complex process that occurs in four overlapping stages: haemostasis, inflammation, proliferation, and remodelling (Barrientos et al., 2008; Guo & Dipietro, 2010; Morton, 2016). Initially, haemostasis involves the formation of a blood clot to prevent bleeding. This is followed by inflammation, where immune cells combat infection and clear debris. In the proliferation phase, new tissue and blood vessels form, aiding in wound closure. Finally, remodelling strengthens the newly formed tissue over time. When wounds become infected, this orderly process can become disrupted, leading to prolonged inflammation and delayed healing. The formation of biofilms within wounds protects microbial communities from immune responses and treatments (Cooper et al., 2014; James et al., 2008; Percival et al., 2015; Wu et al., 2019; Zhao et al., 2013), further impeding the proliferation and remodelling phases. This disruption not only extends the healing time but can also increase the risk of complications.

Wound infections occur when pathogenic microorganisms invade and proliferate within the wound. The source of these infecting microorganisms can vary, including the individual's own skin flora, the environment, hospitals/healthcare facilities, and hygienic conditions. The body's immune system is equipped to deal with invading pathogens; however, in certain conditions, these infections can evade the body's immune response and disrupt wound healing. Comorbidities such as diabetes, poor vasculature, immunosuppression, hypertension, and poor kidney function can significantly exacerbate wound infections and influence wound healing trajectories (Beyene et al., 2020; Sen, 2021). Wound infections can become chronic, primarily due to the underlying effects of diabetes, which impair the body's ability to heal. The presence of infection further exacerbates this, and delayed healing, poor vascularisation, and the formation of biofilms contribute significantly to the persistence and recurrence of these infections (Ding et al., 2022), making them particularly challenging to treat.

Wounds are home to many microorganisms that together form the wound microbiota. Understanding of the wound microbiota has significantly grown over the last few decades, driven by advancements in technology. Using molecular methods instead of traditional culture-based methods has made identifying a more comprehensive profile

of the wound microbial community possible (Kalan & Brennan, 2019; Liu et al., 2020). While culture-based methods are simple, cost-effective and the common choice of technique in clinical settings, they tend to focus on identifying only a few causative pathogens. This overlooks the highly diverse, complex and polymicrobial nature of wound infections. Multiple culture-based and sequencing-based studies have identified key pathogens such as *Staphylococcus spp.,* especially *S. aureus* and methicillin-resistant *S. aureus* (MRSA), and *P. aeruginosa* as common in DFUs (Bessa et al., 2015; Dowd et al., 2008b; Fazli et al., 2009; Frank et al., 2009; Giacometti et al., 2000; Gjødsbøl et al., 2006; Kalan et al., 2019; Loesche et al., 2017; Mohammed et al., 2017; Rahim et al., 2017; Tipton et al., 2017; Tom et al., 2019; Verbanic et al., 2020). Other species such as *Enterococcus spp., Escherichia coli, Klebsiella spp., Candida spp., Streptococcus spp., Acinetobacter baumannii, Corynebacterium* have also been identified (Bowler et al., 2001; Dowd et al., 2008b; Frank et al., 2009; Gardner et al., 2013; Han et al., 2011; Jneid et al., 2017; Kalan et al., 2016; Misic et al., 2014; Price et al., 2009; Sloan et al., 2019; UK Standards for Microbiology Investigations, 2018; Wolcott et al., 2009). However, many studies employ 16S rRNA sequencing, which has limitations in taxonomic resolution (Nygaard et al., 2020). Advanced long-read sequencing technologies such as Nanopore sequencing can provide deeper insights into the microbial dynamics of DFUs with greater taxonomic resolution.

5.1.1 Sequencing methods

Sequencing technologies have advanced significantly, evolving from time-intensive methods to advanced techniques like Illumina and Nanopore, which offer rapid, high-throughput, and real-time sequencing capabilities. 16S rRNA sequencing has been widely used in the field of microbiome research. This method focuses on the conserved 16S ribosomal RNA gene containing variable regions that can be used to distinguish between bacterial species (Bharti & Grimm, 2021; Weisburg et al., 1991). While the 16S gene is highly conserved, it contains nine hypervariable regions, which provide the basis for identification and classification. While most microbiome studies use the V3-V4 regions for amplicon generation, recent studies have shown that other regions (V1-V3) may be more suitable in specific contexts (Hrovat et al., 2024; Lopez-

Aladid et al., 2023) and sequencing the complete 16S gene provides improved taxonomic resolution than the commonly used V4 region (Johnson et al., 2019). Sequencing the entire gene can capture the full range of variability in the nine hypervariable regions, providing more comprehensive data for higher taxonomic resolution.

PCR amplification of the 16S rRNA gene is typically followed by high-throughput sequencing, such as Illumina sequencing, to generate millions of short-read sequences. These reads are then compared to taxonomic databases to identify and classify bacterial taxa present in the samples. 16S rRNA short-read sequencing provides a taxonomic overview of microbial diversity and has largely become cost-effective in recent times. However, it is limited to bacteria and usually provides taxonomic resolution up to the genera level (Yeo et al., 2024). For example, a study using 16S Illumina sequencing identified *S. aureus* as the commonly sequenced microorganism in infected DFUs, with an average abundance of ~43% in 8 samples, (Malone et al., 2017b). However, in 7 other samples, ~31% abundance was represented by unclassified *Staphylococcus spp.,* with no species-level identification. 16S sequencing also cannot identify viruses or fungi within samples, with the latter having significance in wound infections. Metagenomic shotgun sequencing, performed on the whole genetic material of a sample, can help identify and quantify organisms present in a complex sample. Shotgun sequencing has even enabled the identification of phages in chronic wounds (Verbanic et al., 2022), a previously overlooked microbial component.

Illumina sequencing platforms, by which 16S rRNA and shotgun sequencing are performed, generate short reads (typically up to 300 base pairs) with high accuracy and depth, allowing for the profiling of microbial communities. This method is advantageous for its high throughput nature, cost-effectiveness per base, and wide availability of bioinformatics tools for data analysis. The Human Microbiome Project also used 16S and whole genome shotgun sequencing along with Illumina platforms (Group et al., 2009). However, Illumina sequencing is limited by read length. Nanopore sequencing represents a contrasting approach with its ability to produce long-read sequences in real-time (Deamer et al., 2016). Nanopore devices pass DNA or RNA molecules through a nano-scale pore, measuring changes in electrical current as

nucleotides pass through. This allows for direct sequencing of native DNA or RNA molecules without the need for PCR amplification, offering advantages in terms of speed and portability. Nanopore sequencing generates long reads (with the longest reported read being ~4 Mb), which facilitate higher taxonomic resolution, assembly of complete genomes and the detection of structural variants and complex genomic rearrangements. It is, however, currently associated with higher error rates compared to Illumina sequencing (Stevens et al., 2023). The performance of Illumina and Nanopore platforms has been the subject of extensive comparisons. Some research suggests that both methods yield comparable microbial profiles, with a strong correlation in the taxa identified (Cha et al., 2023; Heikema et al., 2020; Stevens et al., 2023), while others indicate that Nanopore sequencing outperforms Illumina (Nygaard et al., 2020; Szoboszlay et al., 2023; Yeo et al., 2024), providing increased taxonomic resolution. Although Illumina remains the most widely used platform for understanding wound microbiomes, the advantages of Nanopore sequencing, particularly its ability to provide more comprehensive microbial profiles, highlight the potential for further detailed and informative studies.

5.1.2 Diabetic foot ulcers and the diabetic wound microbiome

Diabetes mellitus has a significant impact on wound healing, with estimates indicating that about one-third of people with diabetes will develop a foot ulcer in their lifetime (Edmonds et al., 2021). According to the National Diabetes Foot Care Audit (NDFA) (NHS England, 2023), between 2018 and 2023, approximately 40% of reported ulcers were classified as having ‘severe’ infections. Diabetic foot care in England incurs significant costs to the NHS, estimated at £1 billion per year (The National Institute for Health and Care Excellence (NICE), 2015). Specifically, diabetic foot ulcers (DFUs) are a common and severe complication, with approximately 50% of these ulcers becoming infected, further complicating treatment and recovery (Edmonds et al., 2021). Clinically, DFUs are considered infected if they show the presence of at least two of the following symptoms - local swelling or induration, erythema, local tenderness or pain, local warmth, or purulent discharge (The National Institute for Health and Care Excellence (NICE), 2015). These infections can delay wound healing, lead to a non-healing chronic state and increase the risk of amputation. The NDFA

report (NHS England, 2023) found that ~3% of severe DFU infections between 2018 and 2023 resulted in amputation within six months of the initial expert assessment.

Microbiome studies have revealed that the microbial communities in diabetic wounds are complex and diverse, often harbouring a mix of aerobic and anaerobic bacteria. Specifically, *Staphylococcus* species, including *S. aureus* and MRSA, are considered major pathogens in DFU infections (Lebowitz et al., 2017; Lee et al., 2023; Malone et al., 2013). Other commonly identified microorganisms in DFU infections include *Streptococcus agalactiae* and *P. aeruginosa* (Aziz et al., 2011; Citron et al., 2007; Sadeghpour Heravi et al., 2019). Anaerobic bacteria that have been identified include *F. magna, Prevotella spp., Porphyromonas spp, Anaerococcus spp.* and *Bacteroides fragilis* (Citron et al., 2007; Macdonald et al., 2021; Percival et al., 2018; Villa et al., 2024). A culture-based study found that 43.7% of the wound samples had both aerobic and anaerobic bacteria (Citron et al., 2007), highlighting the presence of anaerobes in diabetic foot infections. In a longitudinal study of DFU microbiomes, which performed 16S sequencing using Illumina technology, Sloan et al. (2019) found that non-healing wounds frequently contained a mix of anaerobes and *Enterobacteriaceae*, while Min et al. (2020) reported an association between an increased abundance of a Gram-positive anaerobic coccus, *Peptoniphilus*, with poor healing. Gram-positive anaerobes also formed the majority group of microorganisms in new and recurrent diabetic foot ulcers (Smith et al., 2016).

Alongside pathogens, various skin commensals have also been reported in DFU microbiomes, and previous studies have indicated a possible role of commensals in promoting healing, priming the immune system, preventing colonisation by pathogens and competing with them in skin diseases and wounds (Parlet et al., 2019; Tomic-Canic et al., 2020). For example, the commensal *Cutibacterium acnes* has been shown to inhibit *S. aureus* biofilm maturation and increase its antibiotic susceptibility (Abbott et al., 2022), and compete with *P. aeruginosa* for carbon sources (Maslova et al., 2024). However, though typically harmless inhabitants of the skin, commensals can become opportunistic pathogens in the context of wounds. Species such as *S. epidermidis* and *C. striatum* usually coexist with their host without causing harm. However, when the integrity of the skin barrier is compromised, the disrupted microenvironment provides an ideal niche for these bacteria to proliferate, leading to

infections that can complicate wound healing. For instance, Wolcott et al. (2016) reported the presence of commensals like coagulase-negative *Staphylococcus, Corynebacterium*, and *Propionibacterium* in the chronic wound microbiota. *C. striatum* is particularly noteworthy since it is known to be a clinically significant pathogen in wounds when it is the predominantly grown species. However, it is likely to be considered a contaminant or part of the normal skin flora in cases where it is not the major species grown. Virgilio et al. (2023) underscored its role in healing outcomes and reported *C. striatum* to be a factor for poor prognosis in hard-to-heal peripheral wounds. This shift from commensal to pathogen is often facilitated by the altered immune response and the presence of other pathogenic bacteria, which together create a complex polymicrobial community.

Previous studies have also explored the antibiotic resistance associated with DFU pathogens. Methicillin resistance has become highly prevalent in wound infections; Wolcott et al. (2016) found that 53% of *S. aureus* strains and 45% of coagulase-negative *Staphylococcus* strains in DFUs carried the *mecA* gene, which confers resistance to β-lactam antibiotics except fifth-generation cephalosporins (Peacock & Paterson, 2015). In a study of *Staphylococci* identified in DFUs, 36% were found to be multidrug resistant (Mottola et al., 2016). This showcases the critical concern associated with antibiotic resistance in DFU microbiomes, and the use of advanced sequencing techniques can enable the identification of resistance genes.

A significant limitation of many DFU microbiome studies is their reliance on 16S rRNA sequencing, which can be addressed by adopting next-generation sequencing (NGS) methods. NGS allows for a more comprehensive analysis of the microbial community. In a longitudinal study over 26 weeks, Kalan et al. (2019) used shotgun Illumina sequencing to obtain species and strain-level resolution of DFU microbiomes and identified *S. aureus* strains that were associated with poor healing outcomes. While Sloan et al. (2019) used 16S sequencing, the authors also applied Nanopore sequencing to a subset of samples and reported comparable taxa identification. However, the authors noted high levels of host DNA, which limited the characterisation of the microbiota. Nevertheless, the use of Nanopore sequencing enabled the authors to identify resistance genes in one subset sample and explore interspecies interactions using metabolic modelling.

Diabetic foot infections present a significant challenge due to their complex microbial communities and high prevalence of antibiotic-resistant pathogens. A deeper understanding of polymicrobial communities in wound infections could inform future treatment strategies. Sequencing-based studies have also previously linked microbial community patterns to wound parameters and healing. Malone et al. (2017b) found that diabetic foot ulcers present for a longer duration (> six weeks) had a more diverse microbial community. In a longitudinal cohort study using 16S rRNA sequencing, the authors found that diabetic foot ulcers with more dynamic microbiomes healed faster (Loesche et al., 2017). This is also supported by Sloan et al. (2019), who reported that the microbiota of healing wounds with specific dominant taxa was more dynamic compared to non-healing wounds. In contrast, Gardiner et al. (2017) found no such correlation in a study with a smaller sample size. Thus, there is a high degree of variability in microbiome studies; hence, no single pattern can easily describe the association between the microbiome and wound healing.

Sequencing-based approaches can shift the focus of wound healing and treatment outcomes from individual causative pathogens to the broader dynamics of microbial communities. The aim of this study was to gain deeper insights into the microbial dynamics within DFUs. By employing advanced sequencing techniques, this research provides a more comprehensive view of the microbial communities in DFUs, moving beyond the limitations of traditional culture-based methods. The novelty of the approach lies in the combination of longitudinal sampling and high-resolution advanced sequencing with the latest technology to track changes in the microbiome over time, correlating these changes with treatment and healing outcomes.

5.2 Aims and Objectives

The aim of this study was to develop a deeper understanding of diabetic foot ulcer microbiomes by employing advanced sequencing techniques. To achieve this goal, a longitudinal clinical study was designed with the following objectives:

- Collect longitudinal wound swab samples from participants with infected diabetic foot ulcers.
- Apply saponin-based host DNA depletion method (as detailed in Chapter 4) to extract DNA from wound samples.
- Sequence the DNA using Oxford Nanopore Sequencing technology.
- Analyse temporal changes in the diabetic foot ulcer microbiome and correlate these changes with treatment responses and healing outcomes for each participant

5.3 Materials and Methods

5.3.1 Study design and sample collection

This study aimed to characterise the wound microbiome of diabetic foot ulcers and changes in the wound microbiome over time. The study was designed to obtain samples in the form of wound swabs from a maximum of 24 participants over a period of five weeks (Figure 5.1). Participants were screened and recruited at the Allam Diabetes Centre (Hull University Teaching Hospitals NHS Foundation Trust) (Study Reference Number 21/YH/0272). Informed consent was obtained from participants prior to recruitment. After informed consent was obtained, the first sample was collected on the same day of recruitment. The wound area was cleaned and debrided as determined by the clinical team, and a clinical team member then collected the swab sample for molecular analysis (flocked swabs, VWR). The clinical team also collected an additional swab to perform routine microbiology analysis as per local clinical diagnostic laboratory processes. Along with sample collection, data on each participant was noted (age, sex, diabetes type). The clinical grade of infection, as defined by the International Working Group on the Diabetic Foot (IWGDF) guidelines, was also noted. After sample collection, the swab for molecular analysis was returned to its original tube and stored in an ice box. On the same day, samples collected for molecular analysis were transported to the University of Hull campus on ice, after which they were stored at -80°C until DNA extraction. Follow-up samples were collected in a similar manner over the next four weeks since the first day of recruitment for each participant. At each appointment, information on antibiotic treatment, wound size and any complications arising related to the wound were noted from clinical records. At the end of the study period, a total of nine participants were recruited to this study (35 samples in total) and data from these nine participants has been presented in this study.

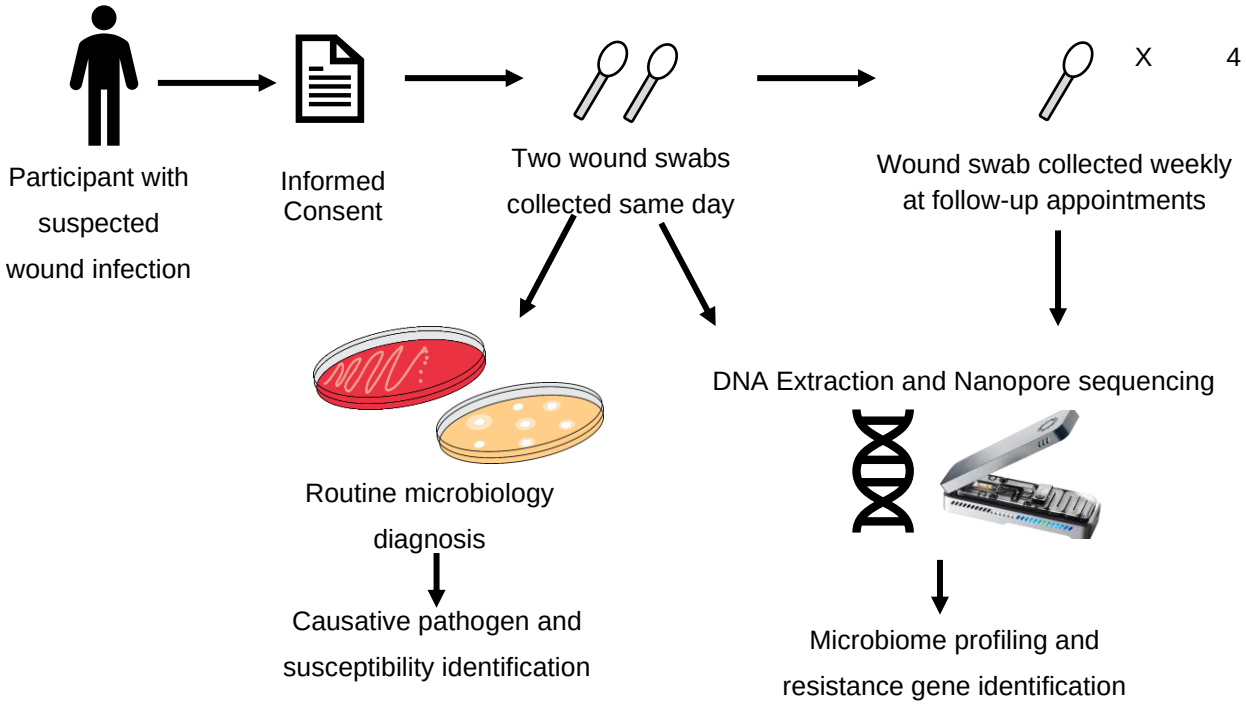

***Figure 5.1: Study design for a pilot longitudinal study of the wound microbiome**, linking to clinical diagnosis and healing outcomes (Study Reference Number 21/YH/0272).*

5.3.2 Swab DNA extraction

Under sterile conditions, the swab head was cut and transferred to a 1.5 mL tube containing 250 µL 1X PBS. The tubes were vortexed for 1 minute in short intervals and centrifuged briefly for 30 seconds to remove any liquid from the lid of the tube. The swab heads remained immersed in PBS for 10 minutes at room temperature. 200 µL of 5% (w/v) saponin was added, and the sample was briefly vortexed and incubated at room temperature. After 10 minutes, nuclease-free water (350 µL) was added to samples, which were further incubated at room temperature for 30 seconds. Samples were then centrifuged at 4°C for 10 minutes at 10,000 rpm. The swab head was removed aseptically and pressed against the tube walls to extract any liquid absorbed by the swab head. The tube was again centrifuged to obtain a sample pellet. The supernatant was discarded, and pellets were resuspended in a solution of 88 µL of 1X PBS, 10 µL buffer 10× Turbo DNAse buffer (Invitrogen AM2238) and 2 µL TurboDNAse (2 Units/µL). This was then incubated for 30 minutes on a thermomixer at 37°C and 1000 rpm to allow for degradation of DNA released from lysed cells. After

30 minutes, 1 mL PBS was added to samples and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was then removed, and pellets were resuspended in 800 µL of Buffer CD1 (the lysis buffer of DNeasy PowerSoil Pro Kit). DNA extraction was performed immediately using the DNeasy PowerSoil Pro Kit method. The manufacturer's recommendations for the PowerSoil kit were followed with minor modifications. The bead-beating was reduced to 5 minutes instead of the recommended 10 minutes, and 50 µL of elution buffer was allowed to incubate in the filter for 5 minutes before the tube was centrifuged and DNA collected. The extracted DNA was quantified using a Qubit 3 Fluorometer HS DNA assay kit. Extracted DNA was stored in Eppendorf DNA LoBind tubes at -20°C.

5.3.3 DNA clean-up

Ampure XP Beads (Beckman Coulter) were used to remove any inhibitors from the DNA prior to sequencing. The manufacturer's recommendations were followed, with minor modifications. During the ethanol washes, 200 µL of 80% ethanol was used. During the elution of DNA from the magnetic beads, buffer C6 from the PowerSoil Pro Kit was used. DNA was stored in Eppendorf DNA LoBind tubes at -20°C.

5.3.4 DNA sequencing

DNA sequencing was conducted using the University of York Genomics Services. Nanopore sequencing with super-accuracy basecalling was performed using a PromethION flow cell and Rapid PCR Barcoding Kit (SQK-RBP114.24) from Oxford Nanopore Technologies. The parameters and versions used are summarised in the table below (Table 5.1).

Table 5.1: PromethION Sequencing Parameters and Software version

Run Settings	Parameter/Software version
Flow cell type	FLO-PRO114M
Run limit	72 hours

Run Settings	Parameter/Software version
Minimum read length	200 bps
Basecalling	On, Super-accurate basecalling, 400 bps
Guppy	7.1.4
MinKNOW	23.07.12
MinKNOW Core	5.7.5
Bream	7.7.6
Configuration	5.7.11

5.3.5 Bioinformatics

Basecalled data (.fastq) was obtained from the University of York Genomics Services. The basecalled data underwent quality filtering using prinseq-lite (v0.20.4) with a dust score cut-off of 7. The filtered data was then subsequently mapped to the human genome (GrCh38) to remove human reads using minimap2 (v2.15) and samtools (v1.3.1). A Snakemake workflow (v1.2) developed at the University of Hull was used for further data analysis. Within this workflow, data filtering employed the default minimum length requirement of 100 bps and a Phred quality score of 7. Taxonomic classification was performed using Kraken2 (v2.1.3), applying the default parameters in the workflow (threshold score of 0.05 and minimum score parameter of 10). A custom database was built for Kraken2 classification, using NCBI taxonomy and a library of archaea, bacteria, fungi, viruses (RefSeq) and human genomes (GRCh38) (downloaded on 09-08-2023). The contig assembly parameter was set to ‘True’ to enable contig assembly using MetaFlye (v2.9.2-b1786). The workflow output files of taxonomic classification (.biom) and contig assembly (.fasta) were used for further analysis.

5.3.5.1 Analysis of Taxonomic Classification

The .biom files were imported into RStudio (v4.2.2). Each PromethION sequencing run included a negative control (an empty swab processed through the same DNA

extraction pipeline). After importing the data into RStudio, all negative control data was subtracted from the respective samples of each run. This was done using an in-house written R code, where the abundance of each OTU detected in the negative control was subtracted from that OTU in all the samples of the same run. Given the differences in library size, rarefaction was performed. The following R packages were used for microbiome data analysis to analyse relative abundance profiles, alpha diversity and beta diversity: *phyloseq, metagMisc, phylosmith, vegan, microbiome*. The alpha diversity of healing and non-healing groups was compared using a Wilcoxon rank sum test. The Bray-Curtis dissimilarity index was compared across healing and non-healing groups, and a Wilcoxon rank sum test was used for statistical significance. The relative abundance of the top 10 species was also compared between healing and non-healing groups, and a Wilcoxon rank sum test was applied. To look at the distribution of Gram-positive and Gram-negative bacteria within the wound microbiome, the AMR package (v1.8.2) in RStudio was used. From the microbiome data, pathogens identified through clinical diagnosis were tracked over time in each participant. To determine changes in the microbiome profile over time for each participant, a Hutcheson t-test was performed to compare the Shannon index of each sample to the sample at the next time point. Data visualisation and statistical analysis were also carried out using RStudio.

5.3.5.2 Identification of resistance genes

The contigs assembled in the workflow were used to identify resistance genes. Antibiotic resistance ontology (ARO) identification was performed using the Resistance Gene Identifier (RGI main, CARD database v3.2.7) with the –low-quality flag. Only strict and perfect hits were included. ARO hits were further filtered to include only those with at least 95% identity match to the top match in CARD. Further, only distinct genes were included to result in the number of unique genes per sample. Distinct or unique genes refer to all individual genes detected within a sample, counted only once, even if they appear multiple times. K-mer Prediction of Pathogen-of-Origin was also carried out for ARG hits using the default 61 bp k-mers CARD database and the default minimum coverage of 10.

5.4 Results

5.4.1 Participant demographics and wound characteristics

At the end of the clinical study sample collection period, a total of nine participants had been recruited for the study (Table 5.2), between the age range of 34-64 years. Of the nine participants, 4 were females and 5 were males. In total, 37 samples were collected from nine participants. For each participant, diabetes type was noted, and the clinical team evaluated the wound infection to determine the grade of infection (Lipsky et al., 2019). Two participants had type 1 diabetes, while the remaining seven had type 2 diabetes. Two of the participants had a Grade 1 infection, while others were found to have a Grade 2 infection.

Table 5.2: Participant demographics, infection grade and sample collection

Participant	Sex	Age (in years)	Diabetes Type	Clinical Grade of Infection	Number of Samples Collected
1	Male	60	2	Grade 2	3
2	Female	48	2	Grade 2	5
3	Female	47	2	Grade 2	2
4	Male	45	2	Grade 1	5
5	Female	34	1	Grade 2	5
6	Male	64	2	Grade 2	5
7	Male	56	2	Grade 2	3
8	Female	41	1	Grade 1	4
9	Male	51	2	Grade 2	5

At each sample collection time point, wound size (length, width and depth) was measured for each participant. This information was used to identify changes in wound dimensions over time (Figure 5.2A). To identify wounds as ‘healing’ or ‘non-healing’,

each week's wound area (length x width) was calculated as a percentage of the wound area at week 1 (Figure 5.2B). A more than 50% change in wound area (from the first week of sample collection to the last available sample for each participant) was considered a predictor of healing (Coerper et al., 2009; Snyder et al., 2010). Based on this parameter, participants 1, 2, 5, 8 and 9 were found to have ‘healing’ wounds and participants 3, 4, 6, and 7 were found to have ‘non-healing’ wounds.

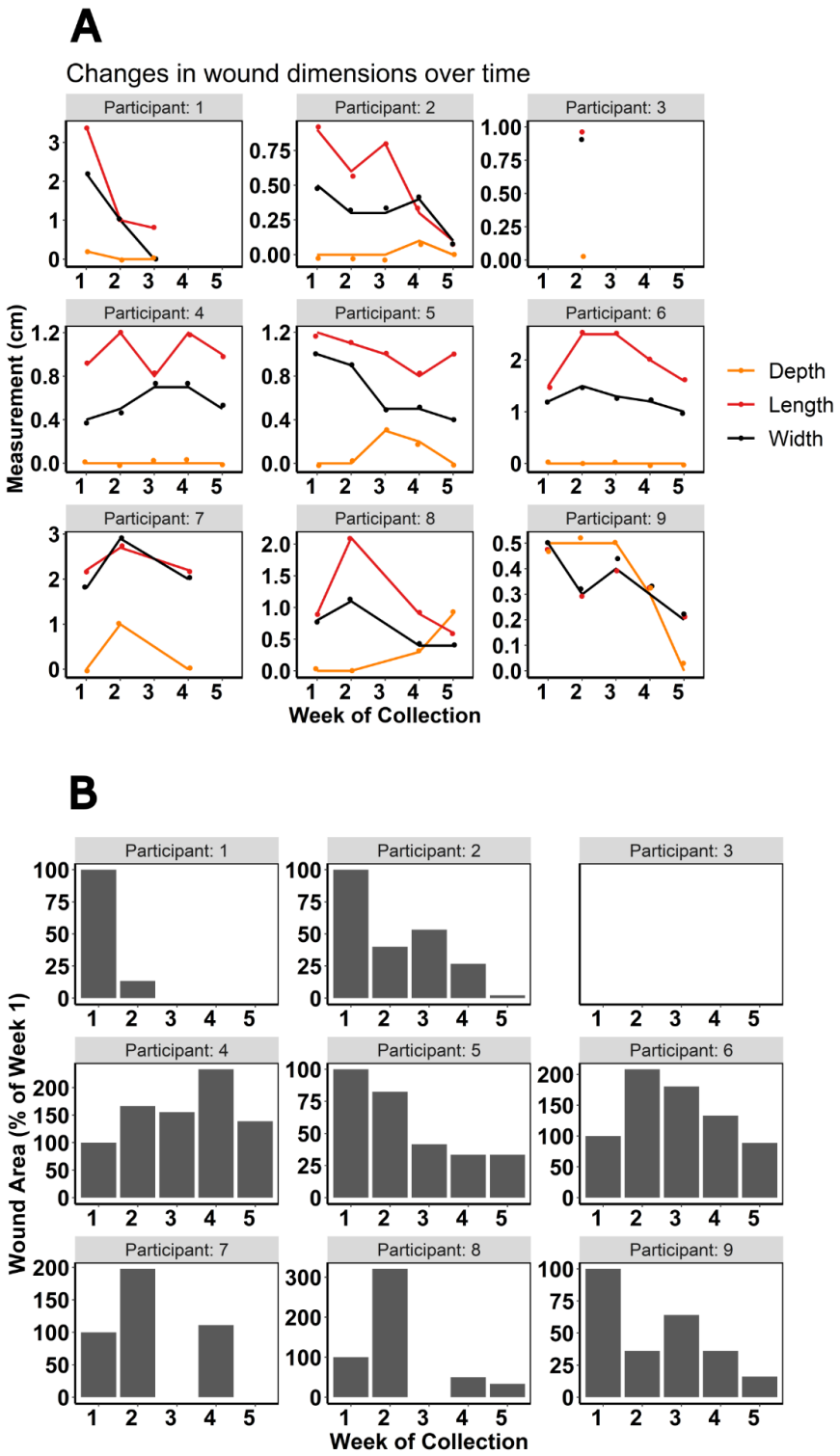

Figure 5.2: Wound area measurements of DFUs. *(A) Changes in wound dimensions over time for each participant. (B) Wound area (length x width) percentage over time for each participant.*

5.4.2 DNA was extracted at extremely low quantities for certain clinical samples

All samples collected were subjected to DNA extraction with the 5% saponin-based host DNA depletion method (see Chapter 4). Certain samples yielded extremely low levels of DNA (Table 5.3), detected as non-quantifiable on the Qubit fluorometer. These samples were still included in the study and were sent to the Genomics Services at the University of York for PCR-based Nanopore sequencing. The Genomics Services Facility optimised library preparation for these samples, using two additional PCR cycles during amplification in comparison to the other samples and finally ran a separate batch of sequencing on the PromethION device for these low-yield samples. The negative control included in this batch run was then used to normalise this data.

Table 5.3: Samples with low DNA yield

Participant	Samples*
1	3 (P1W1, P1W2, P1W3)
2	5 (P2W1, P2W2, P2W3, P2W4, P2W5)
3	-
4	4 (P4W2, P4W3, P4W4, P4W5)
5	-
6	-
7	1 (P7W2)
8	-
9	2 (P9W4, P9W5)

**The numbers indicate the number of samples with low DNA yield and the information in the brackets corresponds to the sample code (P1W1 = Participant 1 Week 1).*

5.4.3 Comparison of rarefied and non-rarefied data revealed only minor differences at genera level

After DNA sequencing, a large difference in library size was noted for the various samples of this study, with about 10^4 times difference between the smallest (279 reads) and largest (7,900,359 reads) library. Hence, rarefaction was carried out to account for these differences in library size. Given the advantages and disadvantages of performing rarefaction, this study compared rarefied and non-rarefied data to identify any differences at the phyla and genera level. At the phyla level, no differences were noted in the microbiome profiles of each sample in the non-rarefied (Figure 5.3A) and rarefied (Figure 5.3B) datasets.

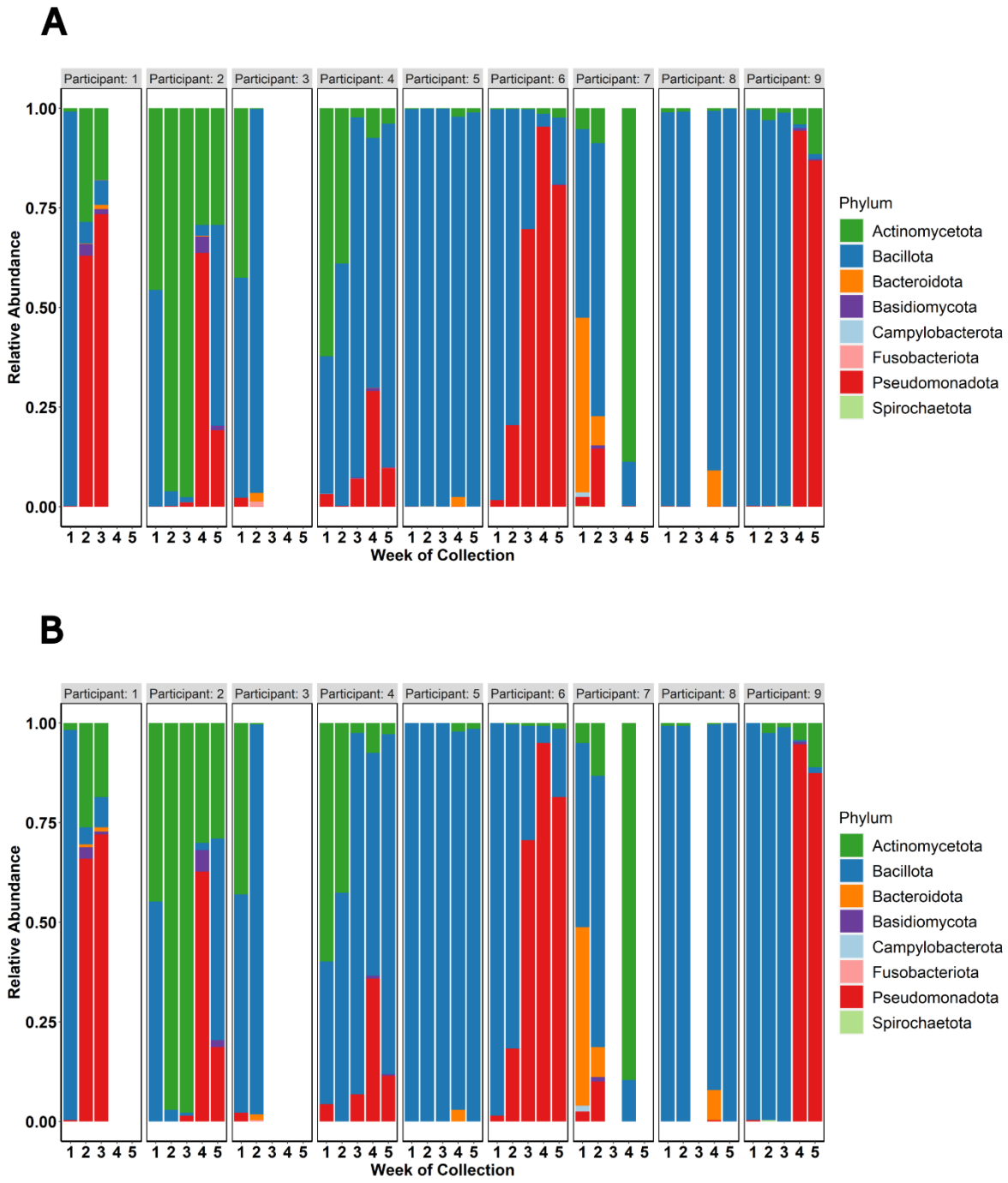

Figure 5.3: Comparison of effects of rarefaction at the phyla-level. *(A) Top 10 phyla for each participant as identified from the non-rarefied dataset. (B) Top 10 phyla for each participant as identified from the rarefied dataset.*

At the genera level, minor differences were observed in the top 10 genera per participant (Figure 5.4). Specifically, three genera that were present in the non-rarefied dataset (Figure 5.4A) of the top 10 genera per participant were absent in the rarefied dataset (Figure 5.4B). The first was *Veilonella*, present only in Participant 1 in the non-rarefied dataset, at very low relative abundance (<1.5%). The second genus was *Fusobacterium*, only present in Participant 3 in the non-rarefied dataset, at very low relative abundance (<1.2%). The third genus that was absent in the rarefied dataset was *Massilia*. This genus was present in the non-rarefied dataset only in samples of participant 2, at relative abundance <0.6%. Further comparisons of rarefied and non-rarefied data at the species level were also made for each participant's samples. This also revealed minor differences; however, these did not represent a large proportion of the wound microbial community. However, given the large discrepancy between library sizes and number of reads observed for samples, rarefaction was necessary to standardise these differences, allowing for fair and unbiased comparisons across samples. Additionally, various statistical tests and biodiversity measures require comparing abundance data, and hence, rarefaction was needed to ensure abundances were normalised.

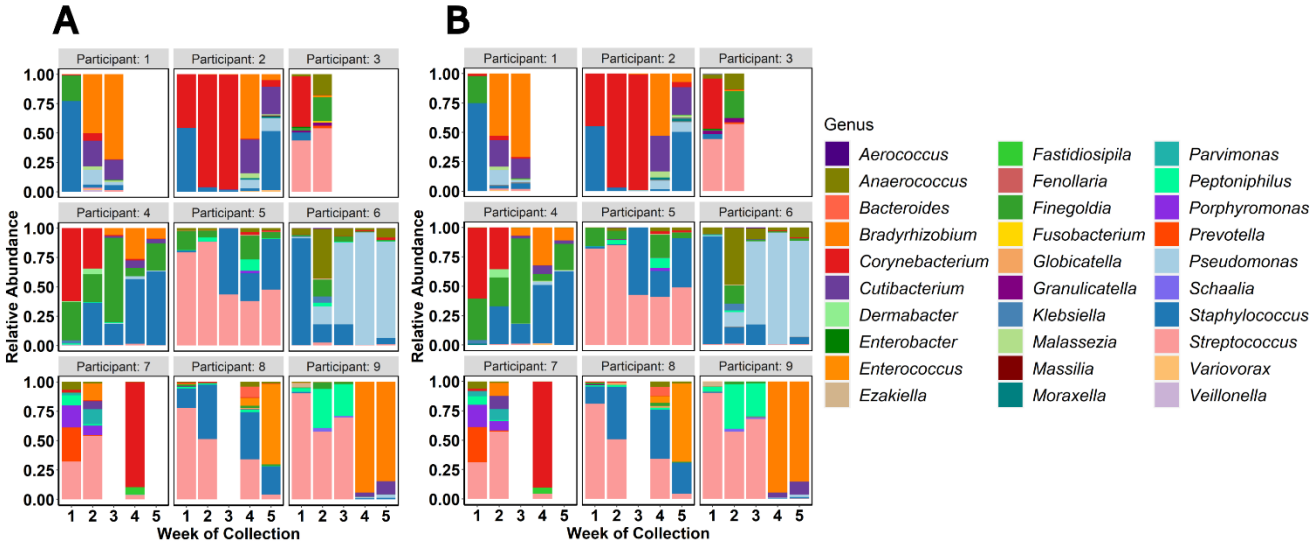

Figure 5.4: Comparison of effects of rarefaction at the genera-level. *(A) Top 10 genera for each participant as identified from the non-rarefied dataset. (B) Top 10 genera for each participant as identified from the rarefied dataset.*

5.4.4 No significant differences were found between the alpha and beta diversities of healing and non-healing wounds

The Shannon alpha diversity index was assessed for healing and non-healing wound groups. A Wilcoxon rank sum test indicated no significant difference (p=0.48) in alpha diversity between these two groups (Figure 5.5). This suggests that the overall richness and evenness of microbial species within samples are comparable regardless of the healing status.

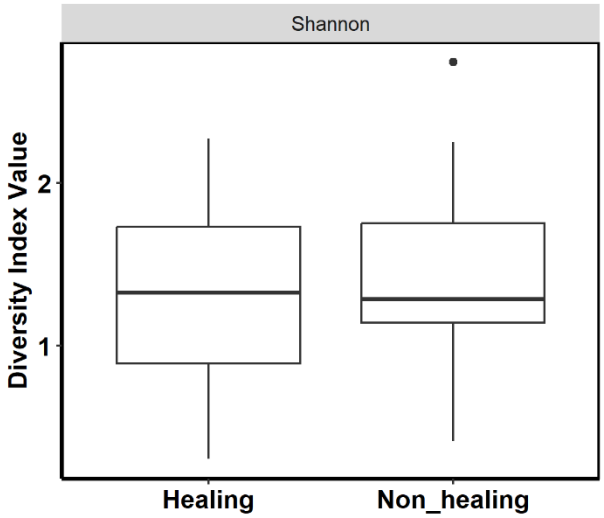

Figure 5.5: Shannon index of alpha diversity in healing and non-healing groups. *Alpha diversity measures the diversity within a single sample, considering both the richness and evenness. The diversity was found to be not significantly different. The box plot shows the median as a central line, with whiskers representing the range within 1.5 times the interquartile range (IQR) from the lower and upper quartiles. Points outside this range are considered outliers.*

Beta diversity, specifically using the Bray-Curtis dissimilarity index, was used to evaluate the differences in microbial community composition between the healing and non-healing groups. The Principal Coordinates Analysis (PCoA) plot suggested that there was some overlap between the two groups, though a PERMANOVA analysis revealed statistically significant differences ($p<0.01$) (Figure 5.6A). Further, considering the first and last samples collected for each participant, the non-healing and healing samples have similarities between the groups but also some differences (though not statistically significant) (Figure 5.6B). However, it is important to note this

study represents a small dataset, with only limited biological replicates, and hence, this might be a limiting factor in understanding 'healing' vs 'non-healing' groups.

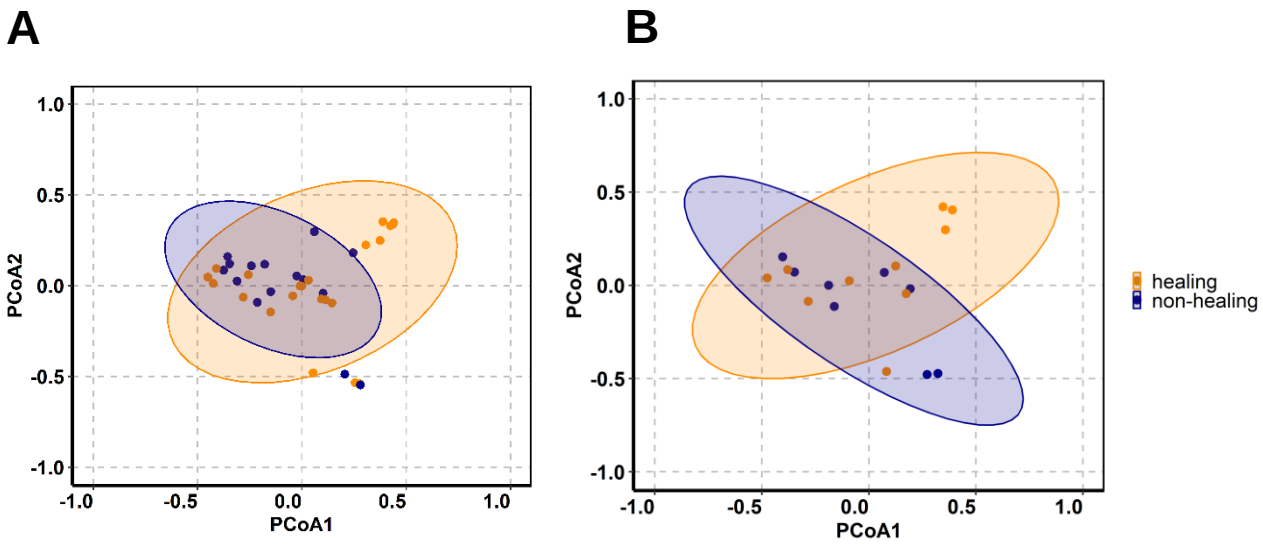

Figure 5.6: Principal Coordinates Analysis (PCoA) plots of healing versus non-healing groups. *Beta diversity was used to evaluate differences in species composition of different groups. PcoA plots were generated based on Bray-Curtis dissimilarity index in (A) all samples and (B) in first and last samples collected for each participant. Each point represents a sample, coloured by group (healing vs. non-healing). Ellipses represent the 95% confidence interval for each group.*

5.4.5 DFU microbiome shows presence of known wound pathogens and two species were significant for healing and non-healing groups

All DFU microbiomes were evaluated to identify the top 10 species in this cohort (Figure 5.7). The DFUs were primarily dominated by Gram-positive bacteria, with 9 out of the top 10 species being Gram-positive. Key known pathogens such as *S. aureus* and *P. aeruginosa*, alongside commensals like *C. striatum, F. magna* and *C. acnes* were detected. *Peptoniphilus*, a Gram-positive anaerobe, was also identified. Further, the relative abundance of these top 10 species in healing and non-healing groups was compared. This revealed that *F. magna* ($p<0.05$) and *P. aeruginosa* ($p<0.01$) had a significantly higher abundance in non-healing groups (Figure 5.8).

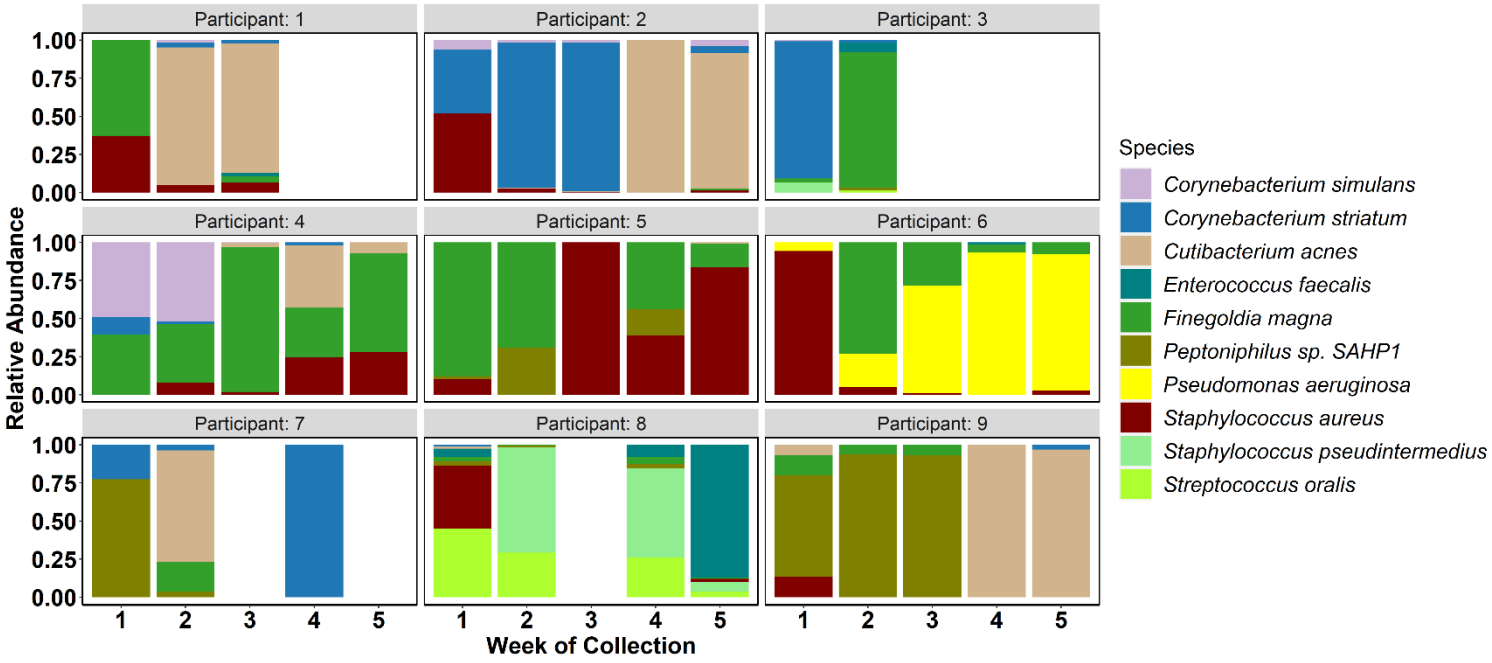

Figure 5.7: Top 10 species identified in diabetic foot ulcers *in this study and their relative abundance over time.*

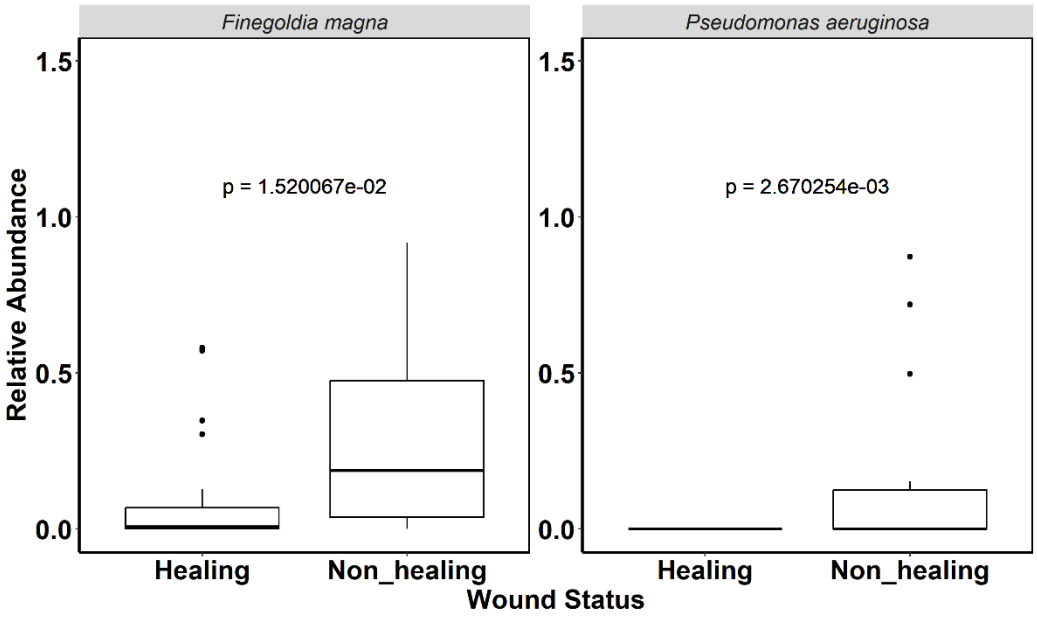

Figure 5.8: Relative abundance of F. magna and P. aeruginosa in healing and non-healing wounds. *The box plot shows the median as a central line, with whiskers extending to 1.5 times the interquartile range (IQR), and outliers shown as points beyond this range.*

5.4.6 Exploring changes in the wound microbiome over time and with treatment

While identifying key microbiome members in the whole cohort was useful, this study aimed to explore changes in the wound microbiome over time and with antibiotic treatment. Given the high diversity of wound microbiomes, the DFU microbiome of each participant was studied in more detail to identify temporal changes and microbial dynamics, taking into account treatment and healing outcomes. For each participant, during the first sample collection, the clinical team collected a wound swab for microbiology diagnosis of clinically significant pathogens and their antibiotic susceptibility. The changes in wound microbiome over time are represented below for each participant, integrating information on pathogen diagnosis, antibiotic treatments and resistance gene identification. While numerous Antibiotic Resistance Ontology (ARO) hits were identified, this study selectively reports on resistance mechanisms, drug classes, and genes that are particularly relevant to each participant's antibiotic treatments and those with known clinical significance. In a subsequent section (5.4.7), ARO data is presented to describe overall trends within the cohort, examining changes in the number of unique ARO hits associated with different resistance mechanisms over time and the shifts in drug classes corresponding to these ARO hits for each participant.

5.4.6.1 Participant 1

Participant 1's wound healed over time (Figure 5.9A), and by week four of sample collection, no open wound was present (hence samples were collected only up to week 3). Nanopore sequencing of the samples revealed a shift in the wound microbiome over time (Figure 5.9B). Clinical microbiology diagnosis identified heavy growth of *S. aureus* in the sample at week 1, with antibiotic susceptibility to flucloxacillin, doxycycline and clarithromycin. Participant 1 was prescribed flucloxacillin as a treatment and had already taken five days of flucloxacillin treatment when the first sample was collected. The causative pathogen (*S. aureus*) had been reported to be susceptible to this drug, and a reduction in its levels was observed over time as the participant received the antibiotic treatment (Figure 5.9C). While microbiology diagnosis identified *S. aureus* as the causative pathogen, microbiome profiling revealed the presence of *Finegoldia magna* at 57% relative abundance (Figure 5.9B).

From week 1 to weeks 2 and 3, a shift in the microbial profile is observed for this participant, with a reduction in levels of *S. aureus* and *F. magna*, the two predominant microbes identified in week 1. *C. acnes,* a skin commensal, largely dominated the microbiome in weeks 2 and 3. The wound microbiome of weeks 2 and 3 also saw the presence of *Corynebacterium tuberculostearicum, Malasezzia*, and *Cutibacterium modestum*, which have been identified as part of the skin flora. Thus, for Participant 1, as the wound healed, the microbiome shifted from being dominated by pathogens to being dominated by a skin commensal. A significant change in Shannon diversity was observed from week 1 to week 2 in the microbiome (Figure 5.9D). Resistance genes were identified only in week 1 for Participant 1, with a larger number of unique hits corresponding to antibiotic efflux mechanisms compared to inactivation mechanisms (Figure 5.9E). A PC1 β-lactamase encoded by *blaZ* was also identified, which is known to be a common source of resistance to penicillins in *S. aureus*, the clinically identified pathogen. While the clinical report did not provide information on antibiotic resistance of the *S. aureus* isolate, various ARO hits identified were traced back to *S. aureus* as the pathogen-of-origin from k-mer-based predictions.

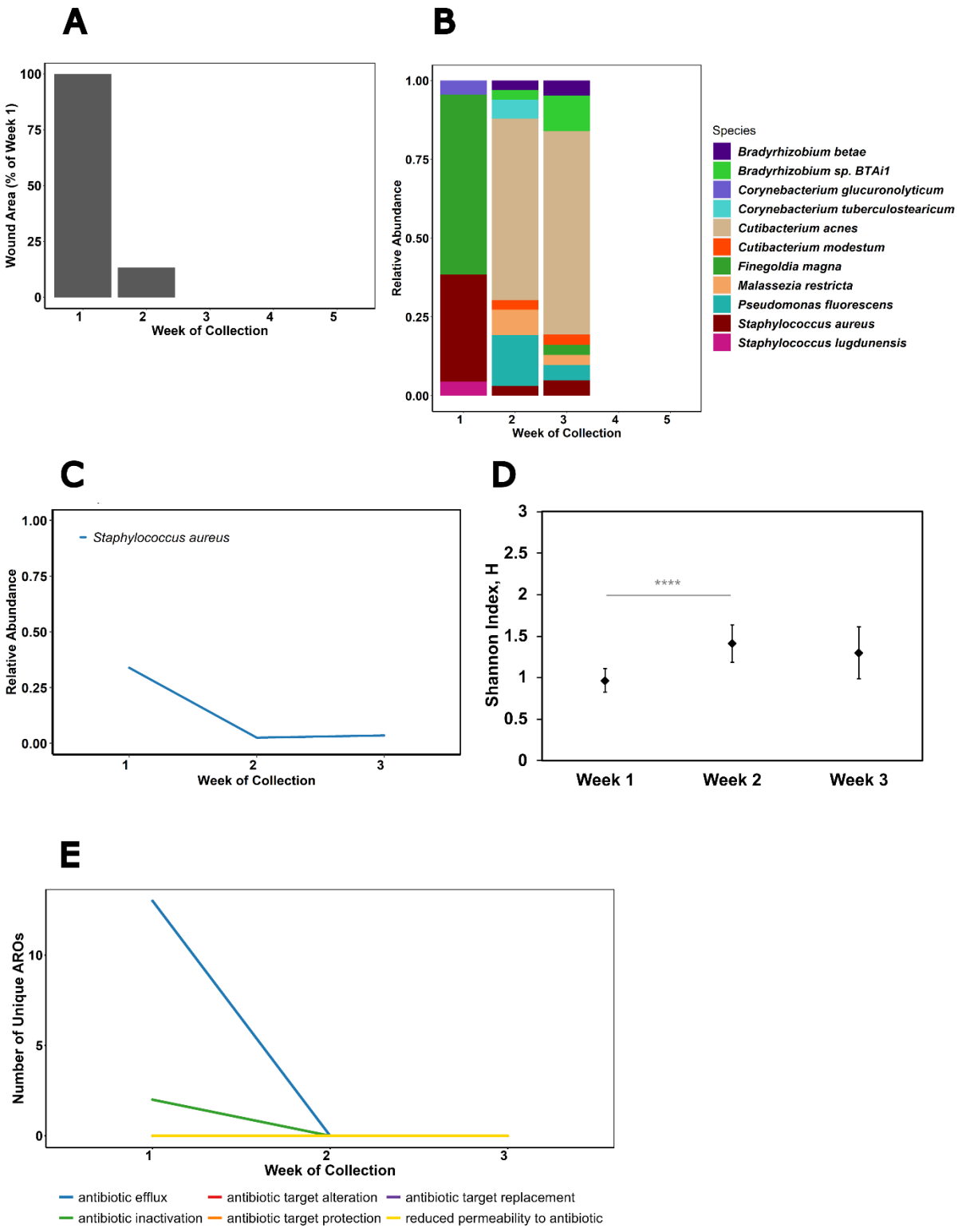

Figure 5.9: Wound microbiome dynamics of Participant 1's DFU. *(A) Wound area percentage over the weeks of collection. (B) Microbiome profile over 3 weeks of collection showing top 10 species. (C) Tracking of the clinically identified causative pathogen of infection. (D) Shannon Index comparisons over weeks of collection (**** p<0.0001). (E) Temporal dynamics of resistance mechanisms detected.*

5.4.6.2 Participant 2

Participant 2's wound was categorised as 'healing' based on wound size and saw a decrease in wound area percentage over five weeks (Figure 5.10A). The wound microbiome at week 1 was dominated by *S. aureus* and *C. striatum* (Figure 5.10B). Participant 2 was not receiving any antibiotics when they were recruited to the study. No wound swab was sent for microbiology diagnosis by the clinical team, and hence, no data was obtained on the causative pathogen and its antibiotic susceptibility. The participant was prescribed doxycycline at their appointment during week 1 for the entire duration of sample collection. In weeks 2 and 3, *S. aureus* levels underwent a reduction, while the microbiome was dominated mainly by *C. striatum*. In weeks 4 and 5, more skin commensals were identified as part of the microbiome, such as *C. acnes*, coagulase-negative *Staphylococcus* such as *S. hominis* and *S. pettenkoferi*, and *Malassezia*. The Shannon diversity significantly changed over time (Figure 5.10D), with week 2 having a significantly lower diversity index than week 1 ($p<0.0001$) and weeks 4 and 5 having a significantly increased diversity index than the respective previous weeks ($p<0.0001$). In the case of Participant 2's DFU, as the wound size reduced, the diversity index increased, accompanied by the presence of more skin commensals. For Participant 2, a temporal decline was seen in the number of unique resistance genes identified corresponding to different resistance mechanisms (Figure 5.10E), with the highest number of AROs corresponding to efflux pumps. Pathogen-of-origin k-mer predictions identified *S. aureus* as the pathogen for various ARO hits, supported by its high presence (~50%) in the wound microbiome at week 1. Interestingly, various ARO hits (*tet(38), tetW, tetA*) belonged to the tetracycline drug class, which corresponds to the antibiotic treatment the participant received (doxycycline).

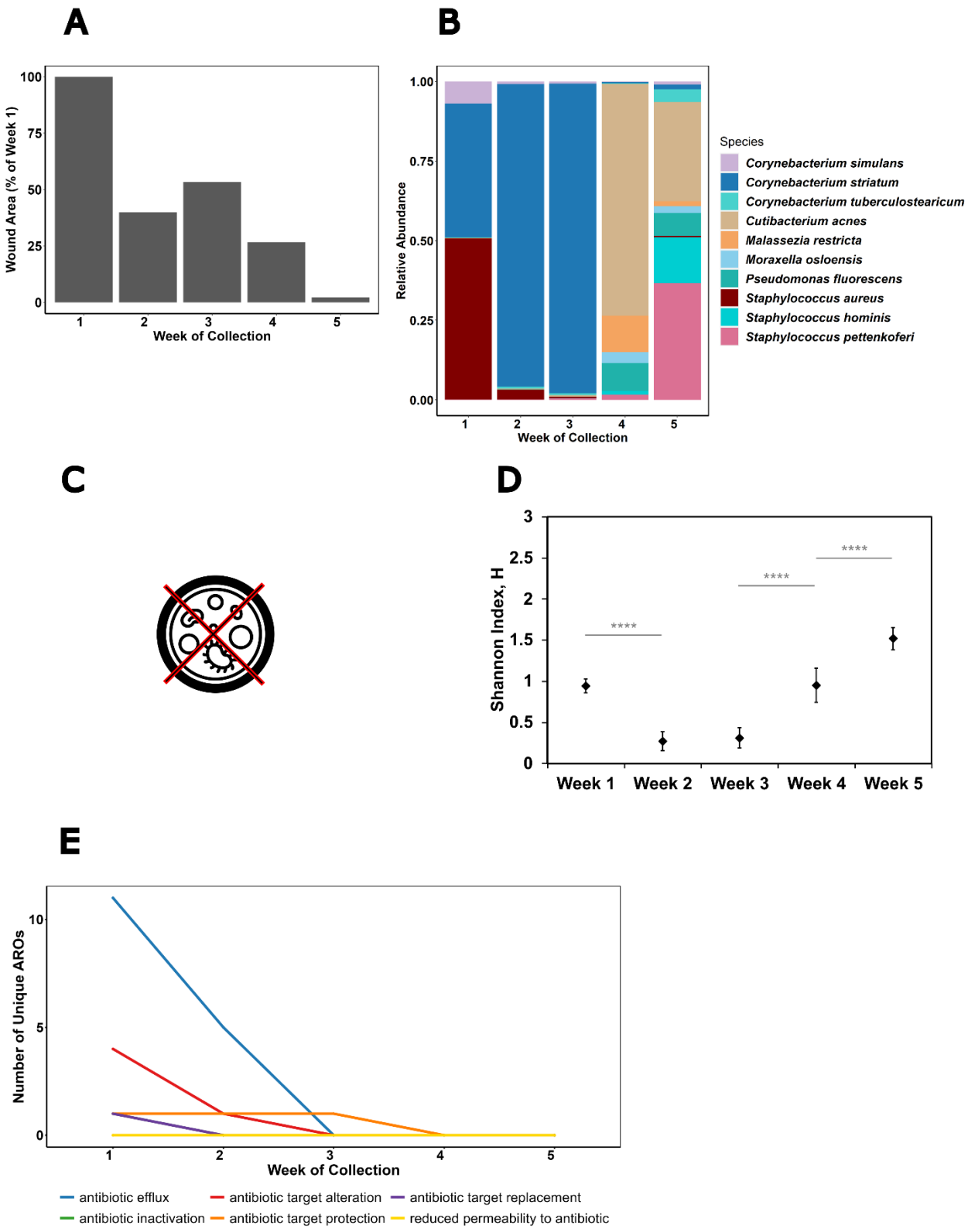

Figure 5.10: Wound microbiome dynamics of Participant 2's DFU. *(A) Wound area percentage over the weeks of collection. (B) Microbiome profile over 5 weeks of collection showing top 10 species. (C) No clinical pathogen identification was obtained for this participant. (D) Shannon Index comparisons of samples over weeks of collection (**** p<0.0001). (E) Temporal dynamics of resistance mechanisms detected.*

5.4.6.3 Participant 3

Participant 3's wound was reported to have been present for three weeks prior to their week 1 clinical visit. The wound size was not measured at week 1 (Figure 5.11A) but was reported to cover the entire top region of the toe. The participant was prescribed co-trimoxazole for two weeks. However, the wound did not heal and led to an amputation of the toe after week 2. The microbiome profile at week 1 showed the presence of pathogens and commensals such as *C. striatum,* facultative anaerobe *S. agalactiae* (Group B *Streptococcus*), *S. dysgalactiae* (Group G *Streptococcus*), *S. epidermis* among others (Figure 5.11B). A shift was observed in week 2, where the microbial flora of the wound was dominated by *F. magna* (Figure 5.11B). Interestingly, the clinical microbiology diagnosis showed heavy growth of Group G *Streptococcus*, Group B *Streptococcus* and *P. aeruginosa,* though their relative abundance in the microbiome was low (Figure 5.11C). *P. aeruginosa* was not detected in the top 10 species of the wound microbiome of Participant 3. In the complete non-rarefied dataset, *P. aeruginosa* was detected in Participant 3's samples at week 1 and week 2, albeit at very low relative abundance (<0.001%). While the microbial profile showed a clear shift (Figure 5.11B), this was not reflected as statistically significant in terms of the Shannon diversity index (Figure 5.11D). This highlights that while diversity indices consider richness and evenness, they do not account for taxonomic identities. Participant 3's data revealed the presence of six resistance mechanisms and had the highest number of drug classes associated with its ARO hits (Figure 5.11E). This participant was prescribed co-trimoxazole, a combination of trimethoprim and sulfamethoxazole. Interestingly, a gene belonging to the dihydrofolate reductase dfr family providing trimethoprim resistance was identified (*dfrC*) in week 1, and another member of the same family, *dfrE* was identified in week 2. Additionally, β-lactamases (OXA, SGV, ACT), methicillin resistance gene *mecA*, as well as other efflux pump genes were also identified in this microbial community.

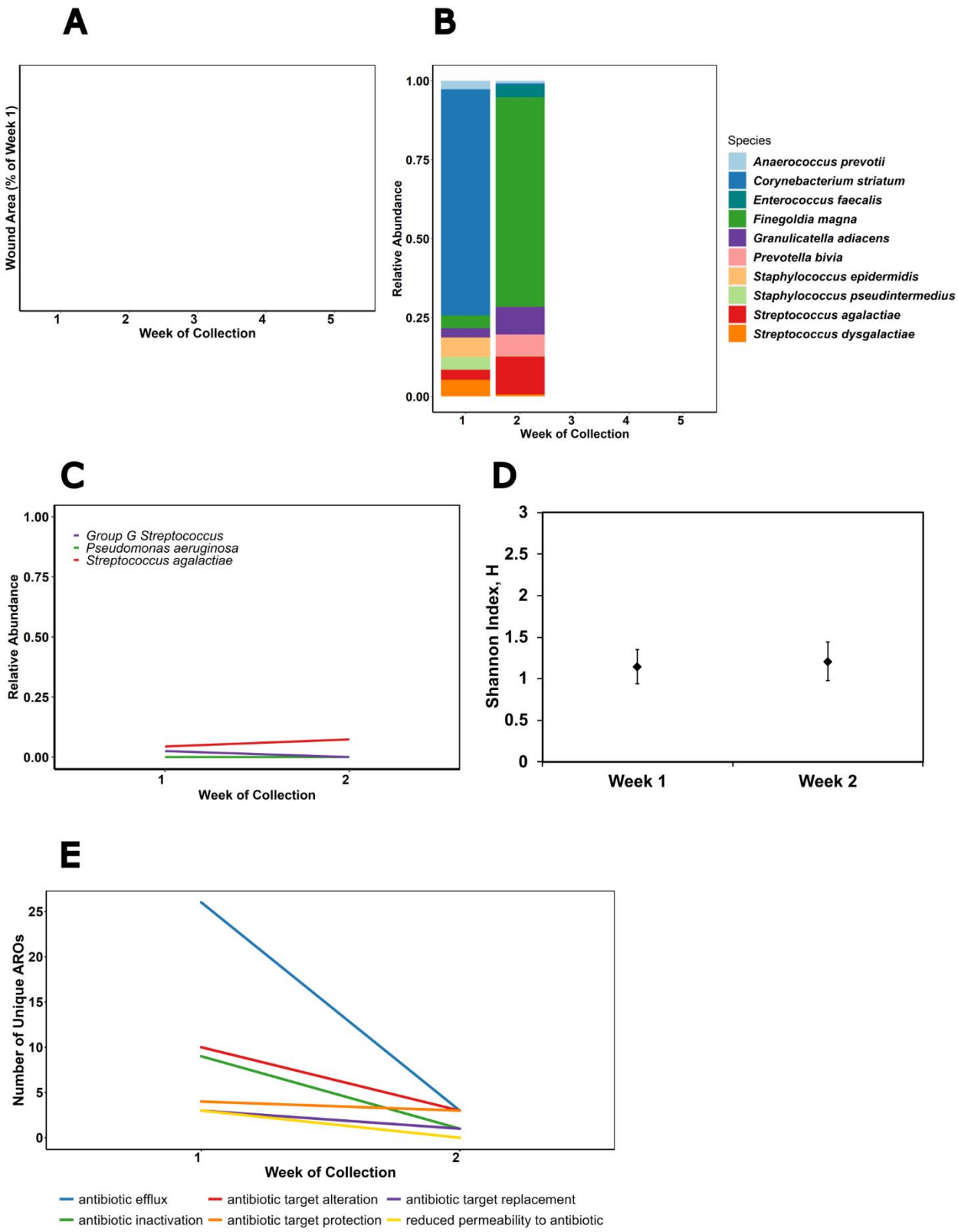

Figure 5.11: Wound microbiome dynamics of Participant 3's DFU. *(A) No wound area measurement available. (B) Microbiome profile over 2 weeks of collection showing top 10 species. (C) Tracking of the clinically identified causative pathogen of infection. (D) Shannon Index comparisons of samples over weeks of collection. (E) Temporal dynamics of resistance mechanisms detected.*

5.4.6.4 Participant 4

Participant 4 had a wound present on their toe for two weeks and had been receiving flucloxacillin for eight days before the first sample collection. At this appointment of week 1, the clinical team decided to switch the treatment prescribed to co-trimoxazole. At the week 2 appointment, the antibiotic prescribed was changed to doxycycline till week 5 in this study. Measurement of wound dimensions revealed that the wound area fluctuated over time (Figure 5.12A). Microbiome profiling revealed that at week 1, *F. magna, C. simulans* and *C. striatum* were the three major species present (Figure 5.12B). While the profile did change over time, a continued presence of *F. magna* and *S. aureus* was observed, along with other *Staphylococcal* species and commensals like *C. acnes*. Interestingly, the clinically determined pathogens, *S. aureus* and *P. aeruginosa,* were not identified within the top 10 species in week 1 (Figure 5.12B). Tracking their levels in the overall microbiome profiles revealed a complete absence of *P. aeruginosa* in the rarefied dataset (Figure 5.12C). *P. aeruginosa* was detected in the complete non-rarefied dataset, thought at low relative abundance (<0.035%). While *S. aureus* was not detected in the week 1 sample of the top 10 species of the rarefied dataset, it was present at ~0.02% in the top 10 species of the non-rarefied dataset. The Shannon diversity index was significantly different through the different weeks of sample collection (Figure 5.12D), and an increase in wound size was found to correspond to an increase in the Shannon diversity index. Fluctuations were seen in the number of unique resistance genes identified, with efflux pump-related genes being the highest in number through the duration of five weeks for Participant 4 (Figure 5.12E). Certain key β-lactamases (*blaZ, bro, tem*), regulators (*mgrA, arlR/S*) and several chromosomal efflux system genes (*norC, norA, sdrM, sepA*) were among the detected resistance genes.

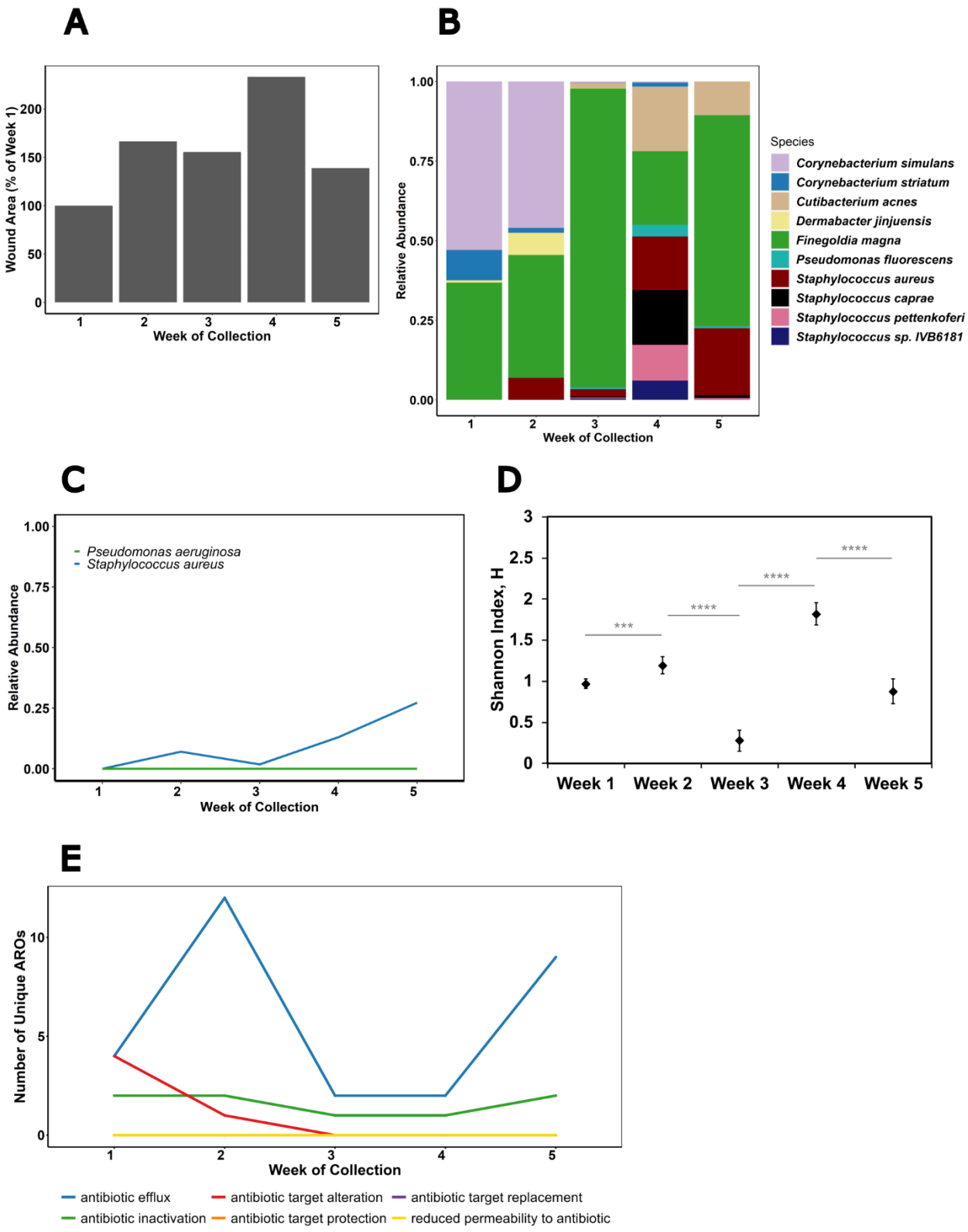

Figure 5.12: Wound microbiome dynamics of Participant 4's DFU. *(A) Wound area percentage over the weeks of collection. (B) Microbiome profile over 5 weeks of collection showing top 10 species. (C) Tracking of the clinically identified causative pathogens of infection. (D) Shannon Index comparisons of samples over weeks of collection (*** p<0.001, **** p<0.0001). (E) Temporal dynamics of resistance mechanisms detected.*

5.4.6.5 Participant 5

Participant 5 had two wounds present on their right foot, and the wounds had been present for two weeks prior to the first week of sampling. The participant had been on doxycycline for three days prior to the first sample collection, and the prescribed dose of antibiotic ended within the first week of this study. Hence, this participant received a short dosage of antibiotic of 8 days and was not taking any antibiotics between weeks 2 to 5 of this study. A gradual decrease in wound area percentage was observed over five weeks (Figure 5.13A), though the wound did not completely close by five weeks. The wound microbiome was found to contain known wound pathogens, such as *S. aureus, S. agalactiae* and *F. magna* (Figure 5.13B). While there were fluctuations in their abundances, these three species accounted for more than 50% of the top 10 species' relative abundance. The clinical microbiology diagnosis identified *S. aureus* and Group B haemolytic *Streptococcus* at moderate and heavy growth levels respectively. *S. aureus* and *S. agalactiae* were identified in the microbiome, and their levels fluctuated through five weeks (Figure 5.13C). While the *S. aureus* strain was reported to be sensitive to doxycycline, an increase in its relative abundance was observed at week 3, which might be correlated with the end of the antibiotic treatment within a short duration. Despite the wound size reducing over the duration of this study, the wound microbiome was still primarily dominated by pathogens *S. aureus, F. magna* and *S. agalactiae* by week 5 (Figure 5.13B). The Shannon diversity index of the wound microbiome significantly changed over the five weeks (Figure 5.13D). A high number of efflux-related resistance genes were detected compared to other mechanisms throughout the sampling period (Figure 5.13E). *S. aureus* and *S. agalactiae* were the predicted pathogen-of-origin for numerous ARO hits, corresponding to their identification as clinical pathogens during diagnosis. Interestingly, the temporal pattern seen for changes in the number of unique AROs (Figure 5.13E) was similar to the temporal pattern seen for the relative abundance of *S. aureus* in the DFU microbiome (Figure 5.13C).

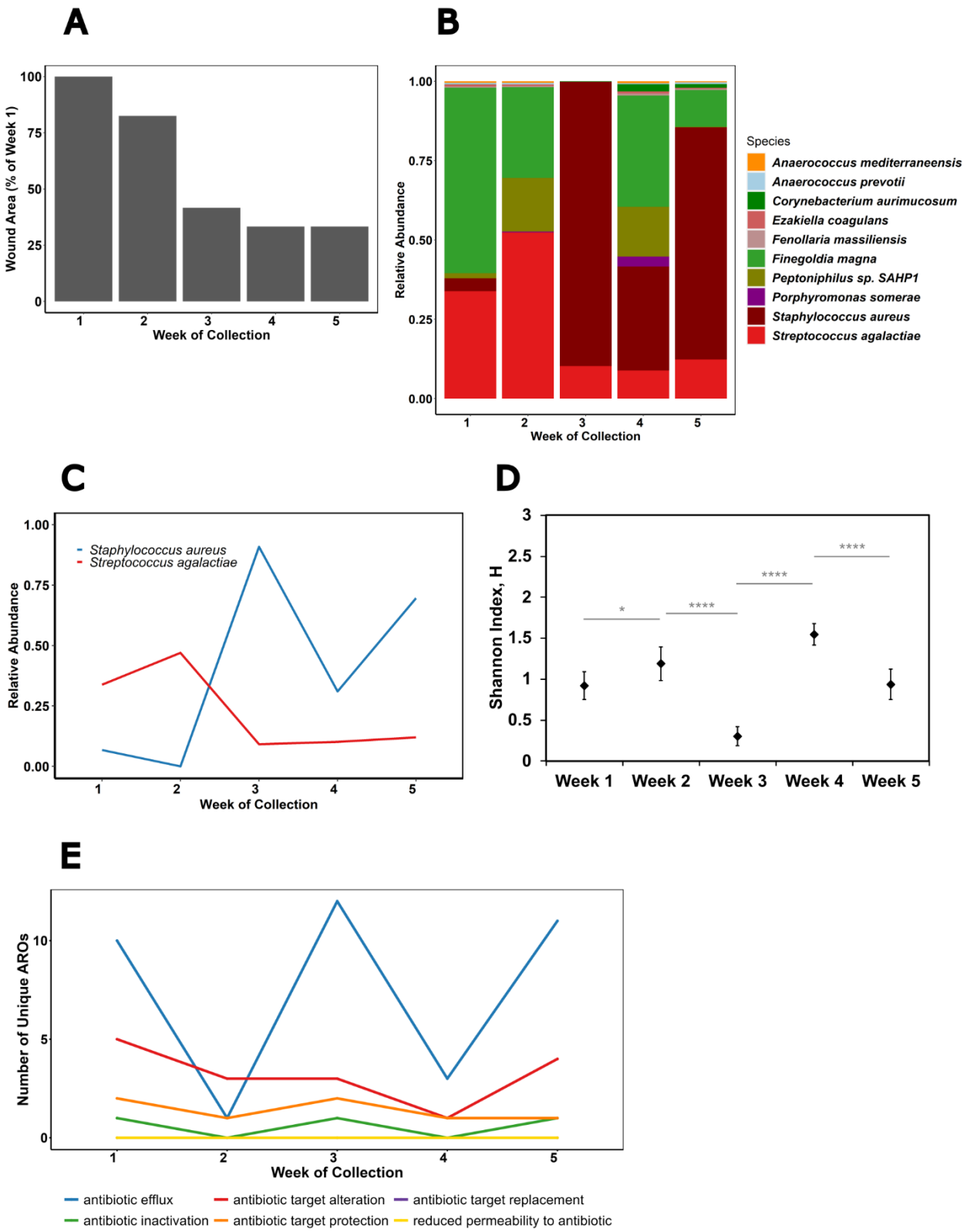

Figure 5.13: Wound microbiome dynamics of Participant 5's DFU. *(A) Wound area percentage over the weeks of collection. (B) Microbiome profile over 5 weeks of collection showing top 10 species. (C) Tracking of the clinically identified causative pathogens of infection. (D) Shannon Index comparisons of samples over weeks of collection (* p<0.05, **** p<0.0001). (E) Temporal dynamics of resistance mechanisms detected.*

5.4.6.6 Participant 6

Participant 6 had a wound on their right toe for eight weeks prior to the day of the first sample collection. The participant was prescribed doxycycline on the day of sampling of week 1 and the prescription continued throughout five weeks. Over the duration of the five weeks, the wound size fluctuated and increased in size in comparison to week 1 (Figure 5.14A). The microbiome profile also changed over time (Figure 5.14B), with the wound showing a high relative abundance of *S. aureus* in week 1. This corresponded with the clinical microbiology diagnosis of *S. aureus* as a causative pathogen (Figure 5.14C). The clinical diagnosis also identified an anaerobe present in the sample and the microbiome did indeed show the presence of anaerobes such as *A. obesiensis, A. prevotii* and *F. magna*. The clinical diagnosis report indicated that *S. aureus* was sensitive to doxycycline, the antibiotic prescribed to this participant, and indeed a sharp decline in the relative abundance of *S. aureus* was seen after week 1. The increase in wound size at week 2 (Figure 5.14A) corresponded to a change in the microbiome profile, with the microbiome at week 1 being primarily dominated by *S. aureus*, and in week 2 by *F. magna*. However, this did not correspond to a significant difference in alpha diversity (Figure 5.14D). Interestingly, over time, the relative abundance of *P. aeruginosa* increased (Figure 5.14B); however, this wound pathogen was not reported in the clinical diagnosis despite its low relative abundance in week 1. *Staphylococcal spp.* associated with skin diseases and infections were also present, such as the coagulase-negative strains *S. lugdunensis, S. caprae* and *S. pettenkoferi* (Figure 5.14B). The Shannon diversity index also changed over time (Figure 5.14D). A decrease in the number of unique AROs was seen over time (Figure 5.14E). An interesting group of genes that were identified in samples of Participant 6 include the *mex* efflux pump genes (*mexA, mexB, mexR, mexW*), which were not identified in any other DFUs in this study. K-mer predictions identified *P. aeruginosa,* which was present in the microbiome, as the pathogen-of-origin for these genes.

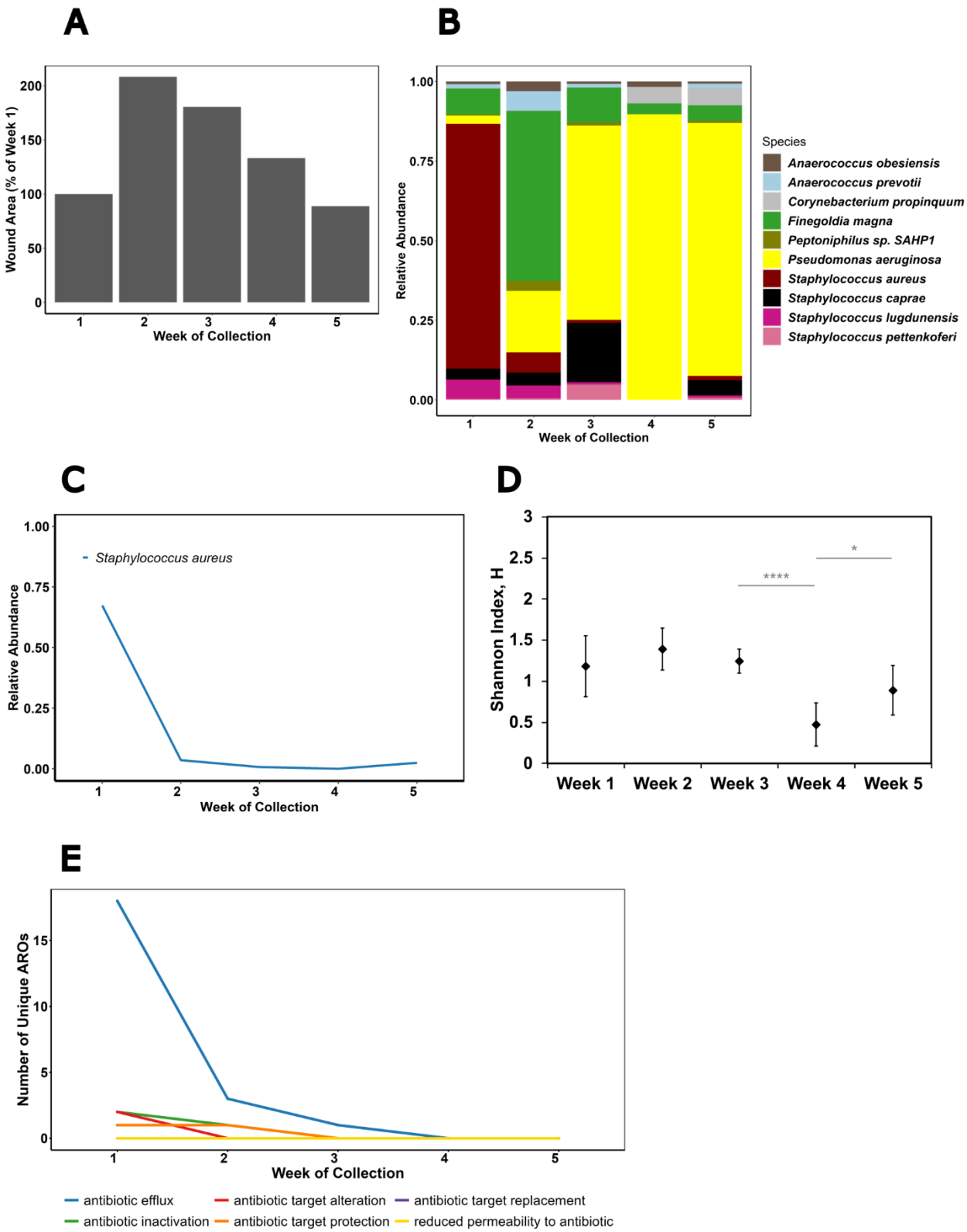

Figure 5.14: Wound microbiome dynamics of Participant 6's DFU. *(A) Wound area percentage over the weeks of collection. (B) Microbiome profile over 5 weeks of collection showing top 10 species. (C) Tracking of the clinically identified causative pathogens of infection. (D) Shannon Index comparisons of samples over weeks of collection (* p<0.05, **** p<0.0001). (E) Temporal dynamics of resistance mechanisms detected.*

5.4.6.7 Participant 7

This participant had a wound present for six months prior to the first week of sample collection in this study. On the day of sample collection at week 1, the wound size was ~3.96 cm^2 (Figure 5.15A). The participant was prescribed co-trimoxazole (the participant was hospitalised, and antibiotic treatment was provided via IV route initially). During week 2, the participant was still hospitalised; hence, the clinical team collected the sample at the ward. The wound size had increased to ~7.83 cm^2 and the wound depth had increased leading to exposure of the bone. By week 3 the participant had been discharged from the hospital, but no data was obtained in week 3 due to a COVID-positive diagnosis. By week 4, the wound size had reduced to 4.4 cm^2 but was still larger than the wound size at week 1. At week 5, the participant had been hospitalised for other medical reasons; hence, no sample or data was obtained. The microbiome profiling revealed that at week 1, the top species in the wound were anaerobes such as *Porphyromonas, Prevotella, Parvimonas* and *Peptoniphilus* (Figure 5.15B). In week 2, a change in the microbiome profile was noted, with differences in the relative abundance of the anaerobes and the presence of *C. acnes*. In week 4, the profile was significantly different, dominated by *C. striatum*. The clinical diagnosis identified heavy growth of Group G haemolytic *Streptococcus*, which was tracked in the microbiome dataset *as S. dysgalactiae* (Figure 5.15C). The pathogen was reported to be sensitive to co-trimoxazole, and this corresponded to a decline in the relative abundance of *S. dysgalactiae* with treatment (Figure 5.15C). However, this heavy growth could have masked the identification of other pathogens of interest, such as *C. striatum*, the relative abundance of which is seen to increase in week 4 (Figure 5.15B). Despite the differences seen in the microbiome at weeks 1 and 2, this did not reflect as a statistically significant difference in the Shannon diversity index (Figure 5.15D), indicating that diversity measures are not always reflective of differences in the microbiome between samples. The Shannon diversity index at week 4 was significantly lower than that at week 2. In the case of this participant, despite the reduction in diversity index and wound size at week 4, overall, the wound was not healing in comparison to week 1. Antibiotic inactivation and target protection mechanism-related resistance genes were higher in number in week 1 (Figure 5.15E). While the causative pathogen of Participant 7's wound infection, Group G *Streptococcus*, was reported to be sensitive to co-trimoxazole and the participant was

prescribed the same, genes corresponding to trimethoprim-sulfamethoxazole resistance (*drf* gene family, *sul* genes) and β-lactamases (*cfxA, carb*) were identified in the week 1 sample.

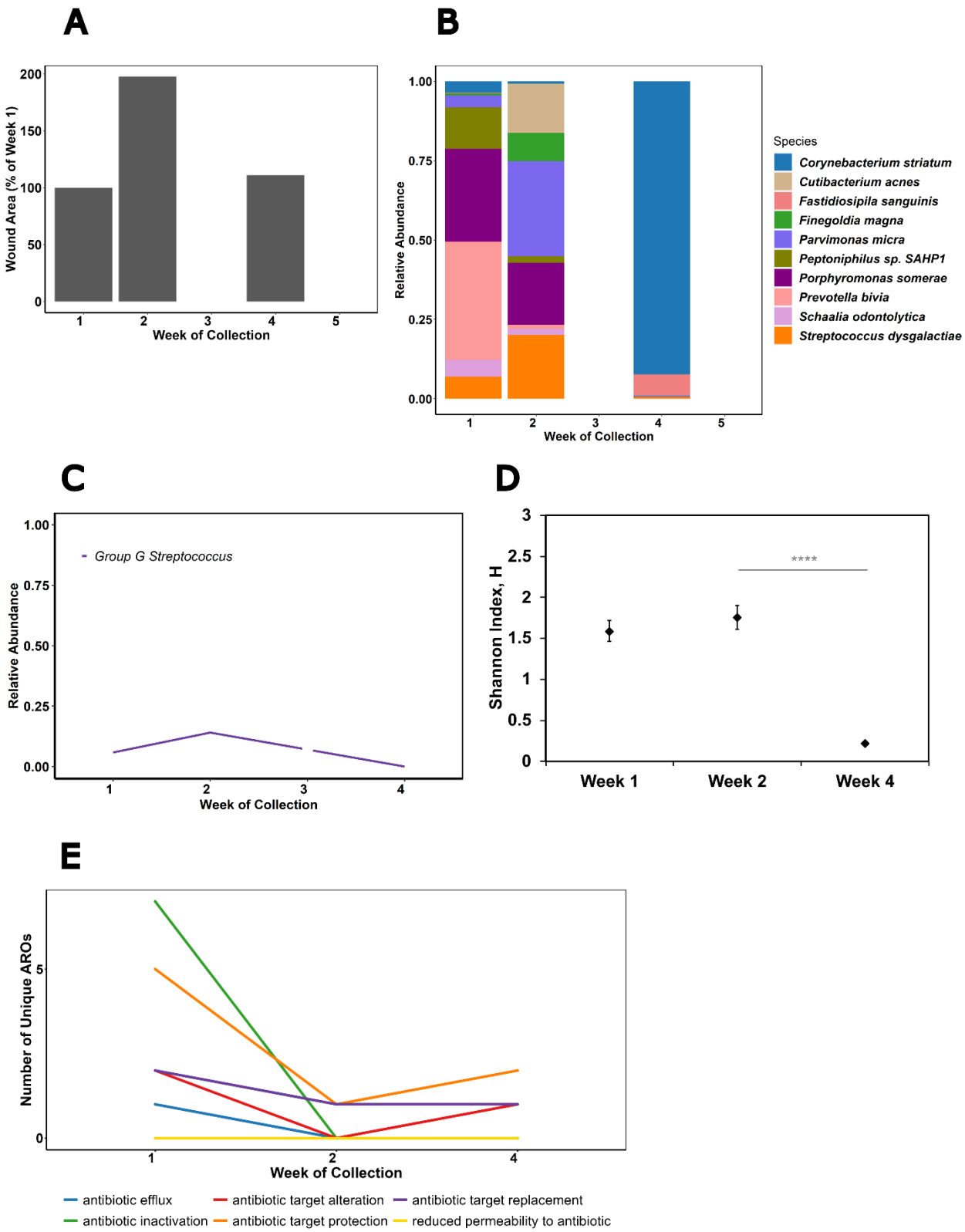

Figure 5.15: Wound microbiome dynamics of Participant 7's DFU. *(A) Wound area percentage over the weeks of collection. (B) Microbiome profile over 4 weeks of collection showing top 10 species. (C) Tracking of the clinically identified causative pathogen of infection. (D) Shannon Index comparisons of samples over weeks of collection (**** p<0.0001). (E) Temporal dynamics of resistance mechanisms detected.*

5.4.6.8 Participant 8

Participant 8 had a wound on their right foot for eight weeks before the first sample collection in this study. At week 1, the participant was prescribed doxycycline to treat the infection. No sample and data were collected at week 3 since no appointment was scheduled at the time. During the initial phase of this study, an increase in wound size was observed for this participant (Figure 5.16A), which gradually decreased over time. While the wound area (length x width) showcases a decrease in wound size (Figure 5.2A), it is important to note that the wound depth increased over five weeks. The microbiome at week 1 consisted of known wound pathogens like *S. aureus* and *F. magna*, as well as skin commensals like *S. epidermidis* (Figure 5.16B). The participant's microbiome also showed the presence of zoonotic pathogens such as *S. pasteurianus, S. canis* and *S. pseudintermedius*. At week 5, the relative abundance of *E. faecalis* had increased, accounting for more than 50% of the composition of the top 10 species in this participant's wound microbiome. The identification of *S. aureus* in the microbiome corresponded with its identification as a clinical pathogen of significance with heavy growth observed. Tracking it throughout the microbiome samples showed a reduction in its relative abundance over the period of five weeks (Figure 5.16C). The Shannon diversity index indicated that the diversity of the microbiome also significantly changed over time (Figure 5.16D). For this participant, no wound swab was collected by the clinical team for microbiology diagnosis at week 1. At week 2, when an increase in wound size was observed, a wound swab was sent for clinical diagnosis. The clinical microbiology diagnosis also revealed that the pathogen identified (*S. aureus*) was resistant to doxycycline. Hence, the antibiotic prescribed to this participant was changed at week 4, from doxycycline to flucloxacillin. Resistance gene identification revealed more unique AROs related to efflux pump mechanism (Figure 5.16E). While no clinical diagnosis of the wound pathogens and their antibiotic resistance profile was made at week 1 for Participant 8, RGI revealed the presence of tetracycline-related ARO hits. This aligns with the clinical diagnosis obtained further in the sampling period, wherein the causative pathogen was reported resistant to doxycycline. Other genes identified (*mepA, mepR, tet(38), norC, lmrS*) represent chromosomally encoded efflux pumps, which are known to play a major role in S*. aureus* virulence and resistance. This is in line with the identification of *S. aureus*

as the causative pathogen in week 2. After the change in antibiotic prescribed at week 4, a decrease in the number of unique AROs was seen at week 5.

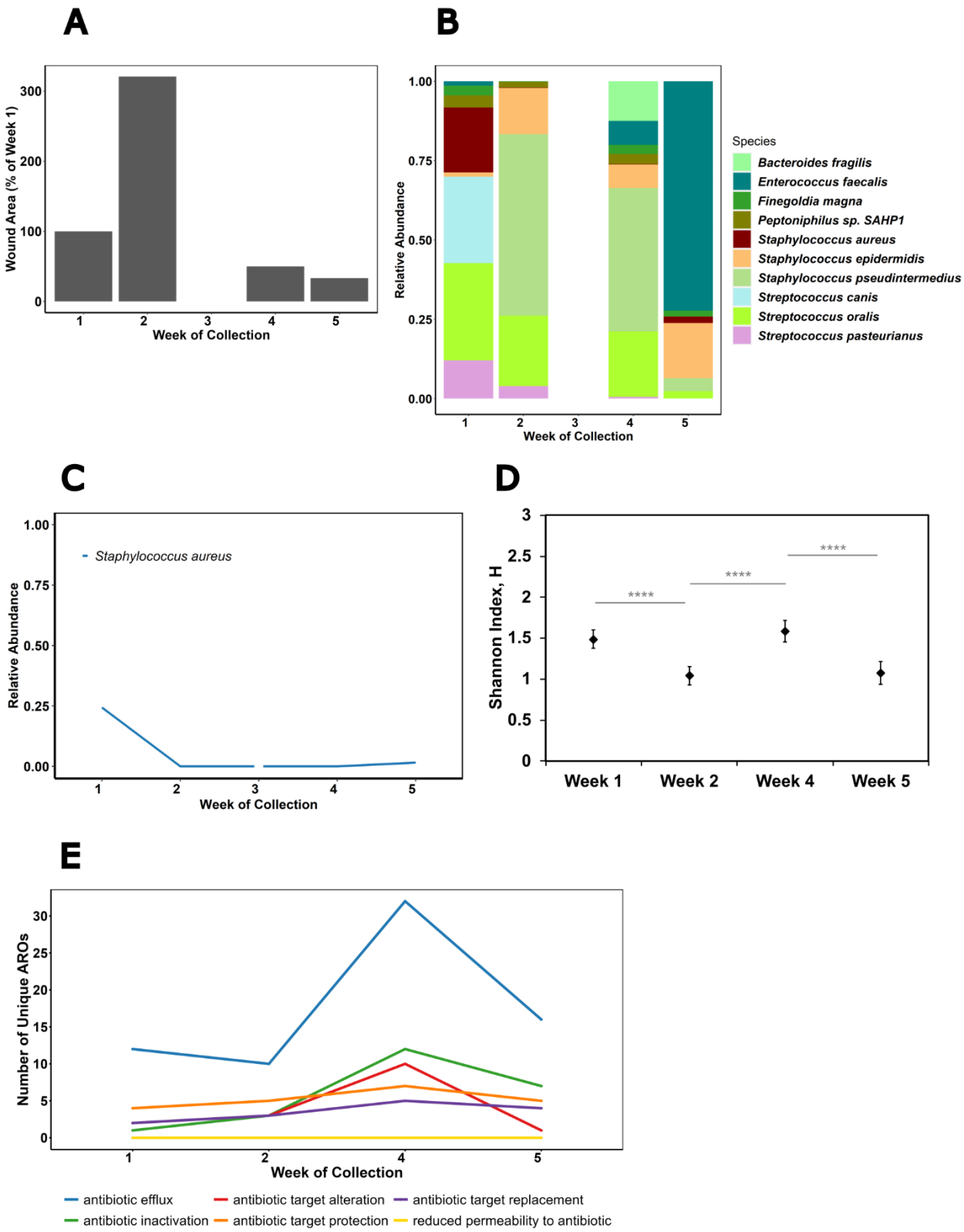

Figure 5.16: Wound microbiome dynamics of Participant 8's DFU. *(A) Wound area percentage over the weeks of collection. (B) Microbiome profile over 5 weeks of collection showing top 10 species. (C) Tracking of the clinically identified causative pathogen of infection. (D) Shannon Index comparisons of samples over weeks of collection (**** p<0.0001). (E) Temporal dynamics of resistance mechanisms detected.*

5.4.6.9 Participant 9

Participant 9 had a wound present on their right foot for six months prior to the first sample collection and had been prescribed doxycycline two days prior. While the wound measured in length and width was <1 cm, the wound was deep and probed to the bone. At week 2, while the wound area reduced (Figure 5.17A), the wound depth was similar to that in week 1 and probed to the bone up to week 3. In the first three weeks, the wound microbiome had a higher relative abundance of anaerobe *Peptoniphilus* (Figure 5.17B). The wound microbiome profile changed drastically in week 4, with a high presence of *C. acnes* and *Bradyrhizobium*. No swab was sent for clinical diagnosis on week 1; a wound swab was collected at week 2 and sent for clinical diagnosis of pathogens. The report indicated the presence of coliform bacilli, moderate growth of Group B haemolytic *Streptococcus* and heavy growth of an anaerobe. Tracking the levels of Group B *Streptococcus* (*S. agalactiae*) showed a gradual decline in relative abundance (Figure 5.17C). The report also showed that the Group B *Streptococcus* was resistant to doxycycline. This information was reviewed by the clinical team in week 3, and the antibiotic prescribed was changed from doxycycline to flucloxacillin. Interestingly, this corresponds with the time at which a drastic change in the microbiome profile was observed (week 4), which also corresponded to a significant change in the Shannon diversity (Figure 5.17D). Temporal dynamics of the number of unique AROs revealed fluctuations over time (Figure 5.17E). Interestingly, a decrease in AROs corresponding to antibiotic target protection, target alteration and efflux-related mechanisms was observed at week 4, alongside an increase of AROs corresponding to antibiotic inactivation. This also corresponds to the time at which a change in the wound microbial community was observed (Figure 5.17B). Samples from the first three weeks for Participant 9 show the presence of tetracycline drug class-associates resistance genes, such as *tet(M)* and *tet(O)*. Indeed, the causative pathogen (*Streptococcus*) was found to be resistant to doxycycline. After the antibiotic treatment was switched at week 3 to flucloxacillin, no *tet* genes were detected at weeks 4 and 5, but penam-related ARO hits were identified.

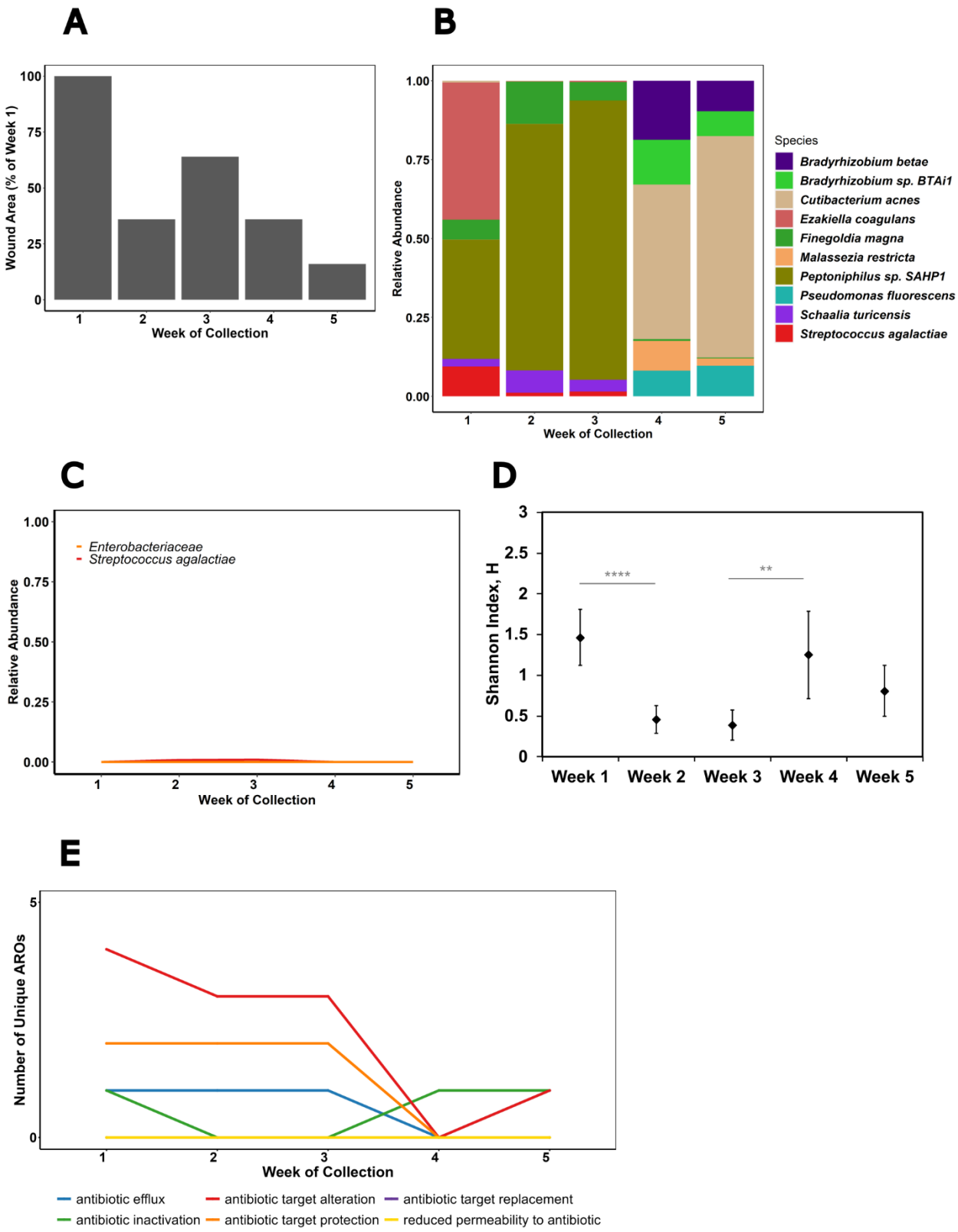

Figure 5.17: Wound microbiome dynamics of Participant 9's DFU. *(A) Wound area percentage over the weeks of collection. (B) Microbiome profile over 5 weeks of collection showing top 10 species. (C) Tracking of the clinically identified causative pathogen of infection. (D) Shannon Index comparisons of samples over weeks of collection (** p<0.01, **** p<0.0001). (E) Temporal dynamics of resistance mechanisms detected.*

5.4.7 Understanding trends in antibiotic resistance genes detected for the entire cohort

Resistance Gene Identifier revealed the identification of various ARO hits, classified into six different resistance mechanisms (Figure 5.18). Overall, the largest number of ARO terms identified belonged to the efflux resistance mechanism in both healing and non-healing groups. The absence of clear differences between healing and non-healing groups suggests that the type of ARO mechanisms does not seem to impact wound healing predictions in this small cohort.

Figure 5.18: ARO hits and resistance mechanisms. *Proportion of non-redundant ARO hits identified for each resistance mechanism in healing and non-healing wounds throughout the duration of this study.*

RGI also enabled the identification of drug classes that the ARO was categorised into, providing further insights into the potential resistance profile of the DFU microbiome (Figure 5.19). The DFU that underwent amputation (participant 3) showed the presence of resistance genes corresponding to 26 different drug classes, the highest among all participants. The results also indicate that for every participant, at least one identified drug class was relevant to the antibiotic treatment they received, and ARO hits corresponding to resistance genes pertinent to the antibiotic treatment were detected. Tetracycline, penam and macrolide resistance genes were detected in all participants.

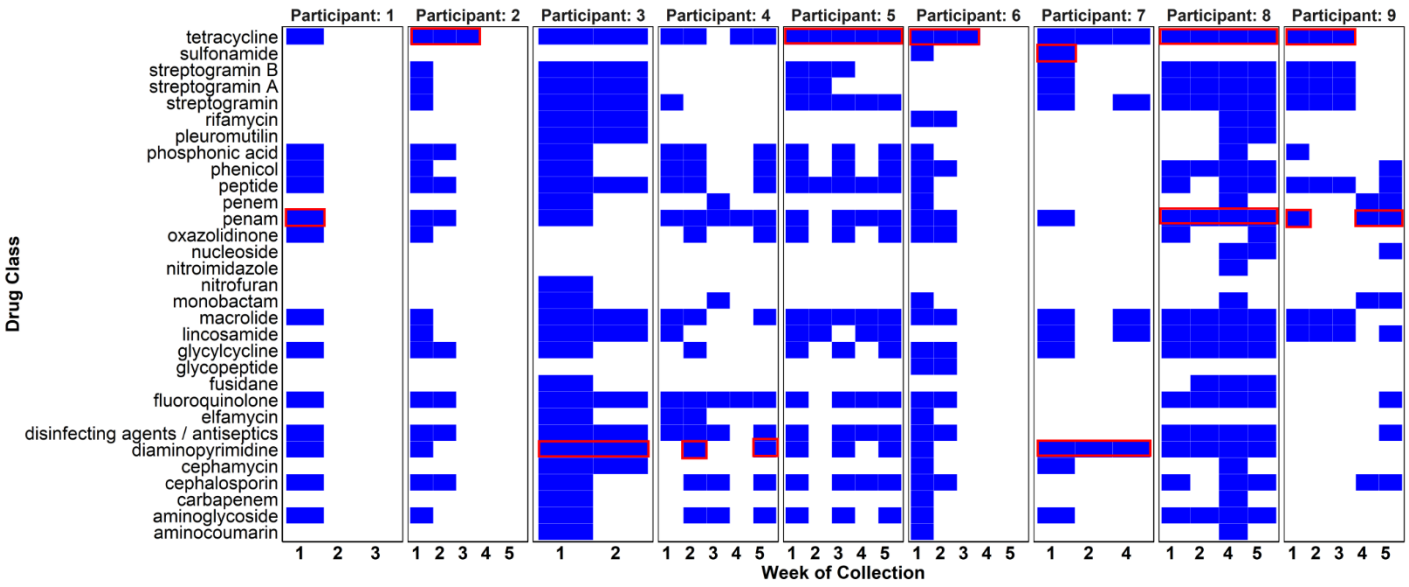

Figure 5.19: Presence of different drug classes based on ARO hits. *Only Perfect and Strict hits with 95% identity match were considered. Blue indicates presence and white indicates absence (absence can be the lack of a sample or the lack of detected ARO terms). The red boxes indicate the presence of ARO terms corresponding to the drug classes administered to each participant throughout the entire study period (flucloxacillin = penam, doxycycline = tetracycline, cotrimoxazole = sulfonamide, diaminopyrimidine)*

Analysis of the mean number of unique (distinct) ARO hits between healing and non-healing wounds indicated no significant differences (Figure 5.20).

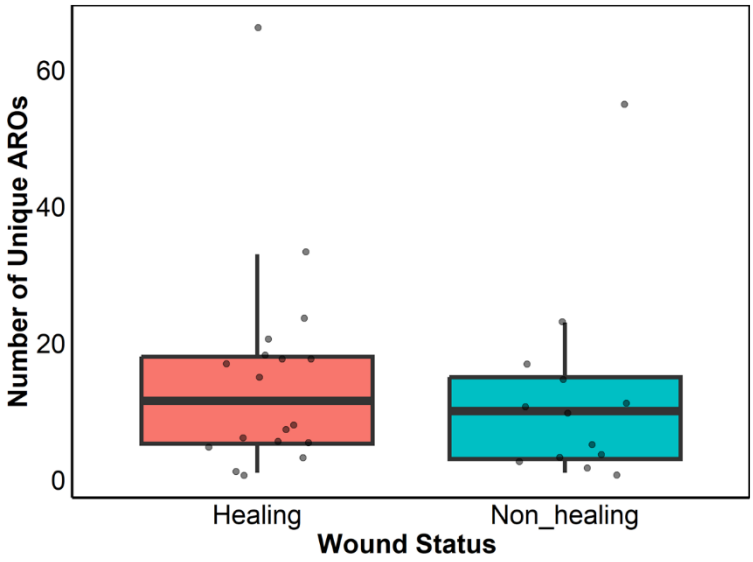

Figure 5.20: Number of Unique AROs in healing and non-healing wounds. *No statistical significance was found when distinct ARO terms were identified and compared across the two groups (Wilcox test, p = 0.37). The box plot shows the median (central line) and the interquartile range (box), with whiskers extending to 1.5 times the interquartile range. Overlayed points represent individual data points.*

5.5 Discussion

Diabetic foot ulcers represent a substantial clinical challenge due to their polymicrobial nature and the high prevalence of antibiotic-resistant pathogens. The microbiota of DFUs is not only diverse but also dynamic, changing over time and in response to antimicrobial treatment interventions. This study's findings align with previous research indicating that DFUs harbour a variety of microorganisms, including both well-known pathogens like *Staphylococcus* and *Streptococcus* as well as others like *Corynebacterium* and *Finegoldia* (Macdonald et al., 2021; Smith et al., 2016). The presence of these microbes, often in biofilm communities which are recalcitrant to antibiotics, underscores the complexity of treating DFUs and the necessity for targeted therapeutic strategies.

The primary aim of this study was to utilise advanced long-read sequencing technology alongside host DNA depletion methods to gain deeper insights into the microbial dynamics within DFUs. This study sought to link microbial community dynamics with treatment and healing outcomes by examining the wound microbiome over time. Additionally, the study aimed to overcome the limitations of traditional culture-based methods and short-read sequencing by providing a more comprehensive and accurate characterisation of the DFU microbiome, including detecting antibiotic resistance genes.

The DFU microbiomes in this study were primarily dominated by Gram-positive bacteria, as has been observed previously (Keogh et al., 2024; Macdonald et al., 2021; Smith et al., 2016). Overall, the top 4 species in DFUs were Gram-positive (*C. striatum, F. magna, S. aureus* and *C. acnes*), which have all been reported as prevalent in DFU microbiomes (Dowd et al., 2008a; Kalan et al., 2019; Loesche et al., 2017).

S. aureus is frequently identified as the predominant pathogen in the microbiomes of diabetic foot ulcers (DFUs). For example, Dowd et al. (2008a) used pyrosequencing and identified *S. aureus* as the predominant species in diabetic foot ulcer samples. Additional studies have consistently demonstrated the high prevalence of *S. aureus* in these infections (Choi et al., 2019; Lebowitz et al., 2017; Lee et al., 2023; Malone et al., 2013). In the present study, *S. aureus* was identified as one of the causative pathogens in 55% of DFUs. However, despite clinical reports indicating ‘heavy’ growth of this pathogen, it was not always the most abundant organism within the microbial

community when assessed using molecular techniques. This discrepancy, frequently observed in this study, suggests that culture-based diagnostic methods may overlook other critical microbial members by favouring well-known and easily culturable pathogens. This emphasises the limitations of traditional culturing techniques and underscores the need for comprehensive molecular approaches to accurately characterise the microbial landscape of DFUs.

Another example of discrepancies between culture-based and sequencing methods was observed in Participant 6. Initially, the causative pathogens identified were *S. aureus* and an anaerobe, which were indeed the predominant species present in the week 1 sample. However, the culture-based diagnosis did not report the presence of the known wound pathogen *P. aeruginosa*, which was detected at approximately 4% abundance by sequencing at week 1. In the subsequent weeks, the levels of *P. aeruginosa* increased to 87%, and the ulcer expanded in size over the four weeks compared to week 1. The high prevalence of *P. aeruginosa* in this DFU also explains why it had significantly higher relative abundance in the non-healing group. Various other species that were not present or present at low abundances in week 1 in all DFUs of this study transitioned from low to high abundance over time, reflecting the dynamic nature of wound microbiomes. Examples include *F. magna* in Participant 3, *C. striatum* in Participant 7 and *E. faecalis* in Participant 8, which went from an abundance of <10% in week 1 to >40% in subsequent weeks. This underscores the importance of not disregarding microbes present in low abundance. Indeed, a previous longitudinal study found that 80% of chronic wound microbiomes contained species that were initially present in low abundances but at later time points became more prominent (Tipton et al., 2017). One plausible reason for this shift could be the antibiotic therapy prescribed, usually targeting a few key pathogens. This could create a niche that allows previously minor species to proliferate. Additionally, changes in the wound environment, such as decreased immune function or altered nutrient availability, could further promote the growth of these low-abundance species. This could potentially impact wound healing outcomes. Species starting in low abundance may go undetected or be deemed clinically insignificant. However, as they become more prominent over time, they could contribute to latent infections that are difficult to treat and could exacerbate the chronicity of the wound. This shift in microbial dominance can complicate treatment and highlight the importance of adaptive,

personalised approaches to wound management, taking into account the evolving microbial community within the wound.

In various DFUs assessed in this study, *C. striatum* and *F. magna* emerged as highly abundant during sequencing, though they were not reported as pathogens in culture-based diagnosis. For example, in the DFU of Participant 3, culture-based diagnosis identified heavy growth of Group G *Streptococcus*, Group B *Streptococcus* and *P. aeruginosa*. However, their relative abundance in the microbiome was low, and a significant proportion of the microbiome was dominated by *C. striatum*. The culture-based diagnostic approach may have allowed the growth of *Corynebacterium*; however, the presence of other predominant pathogens in the sample could have led to its underreporting as a critical pathogen. In the cohort of participants included in this study, *C. striatum* and *F. magna* were, in fact, the most abundant species overall.

Corynebacterium is a commensal of the skin, typically part of the normal flora. *Corynebacterium* can become an opportunistic pathogen, particularly in immunocompromised patients or when the skin barrier is disrupted. Though known to be clinically significant for wound infections, *C. striatum* is often overlooked. Traditional culture-based methods may not always identify this bacterium, especially if it is present in low numbers or overshadowed by the overgrowth of other dominant pathogens. The UK Standards for Microbiology Investigations (2023) recommend that *Corynebacterium* be considered for species-level identification only when it grows equivalent to $>10^4$ CFU/mL in a pure culture. Moreover, it might be dismissed as a contaminant or commensal rather than recognised as a potential contributor to infection, particularly when other key pathogens are grown. The use of molecular techniques, like 16S rRNA sequencing or whole genome sequencing, can help detect *C. striatum* and indeed has revealed its presence in wound microbiomes. In one study, *C. striatum* was identified as the most abundant species (present in 30 samples out of 40) (Dowd et al., 2008b). *Corynebacterium* was also the most commonly identified microbe in diabetic foot osteomyelitis (Johani et al., 2019). In the current study, *Corynebacterium* species were also common, present in 7 out of 9 DFUs. Interestingly, *Corynebacterium* was not reported as a pathogen in the clinical diagnosis of any DFU in this study. The consistent presence of *C. striatum* in wound infections, coupled with the often-dismissed status as a primary pathogen in culture-based diagnosis, suggests the need for traditional diagnostic methods to report *Corynebacterium* as a pathogen.

F. magna is another skin commensal with virulence factors that help it attach and colonise the skin (Murphy & Frick, 2013; Murphy et al., 2014). In certain studies of DFUs, *F. magna* was the most prevalent anaerobe (Citron et al., 2007; Jneid et al., 2018) and has been identified in other DFU microbiome research as well (Choi et al., 2019; Smith et al., 2016). Particularly, *F. magna* was found to have a significantly higher prevalence in wounds with impaired healing (wounds that did not reduce in size by at least 50% in 5 weeks). A similar association with impaired healing was seen for anaerobe *Peptoniphilus* by Min et al. (2020), and for *Finegoldia*, though this was not statistically significant in their study. *F. magna* was the second most abundant species in this study. The presence of anaerobes suggests that the infection has likely penetrated beyond the superficial layers of the skin into deeper tissues (Charles et al., 2015; Gardner et al., 2013), where oxygen levels are reduced. This deeper colonisation can contribute to the chronicity and complexity of the infection. Identification of anaerobes in clinical diagnosis requires specific culture conditions and media (UK Standards for Microbiology Investigations, 2015). Further, as wound infections are polymicrobial, identification of strict anaerobes by culture can be hindered by the overgrowth of facultative anaerobes. Hence, using molecular approaches for diagnosis can be advantageous, as they do not rely on the culturing of microorganisms and can even detect species at lower abundances than traditional culture-based methods. Previous studies have highlighted the vast differences in the prevalence of anaerobes detected by culture-based methods in comparison to molecular techniques (Villa et al., 2024), showcasing how using molecular approaches can improve the detection of anaerobes.

Although earlier research has noted the presence of commensals in wound microbiomes (Tomic-Canic et al., 2020), numerous recent studies are now investigating the potential role of these commensals within polymicrobial communities. The beneficial effects of commensals include skin barrier restoration and wound healing promotion (Harrison et al., 2019; Sachdeva et al., 2022). Newstead et al. (2020) summarised key bacteriocins produced by *S. epidermidis,* which show potential antimicrobial activity against *S. aureus*. However, others have highlighted the harmful effects of commensals. For instance, skin commensals, such as *S. epidermidis* and *Micrococcus luteus*, were seen to enhance *S. aureus* infection in an animal model (Boldock et al., 2018; Gibson et al., 2021). Thus, this potential dual effect of

commensals on wound microbial communities highlights the need to identify such bacteria and not disregard them as commensals.

While the current study focused only on the bacterial component of the microbiome, Kalan et al. (2016) showed that ~80% of diabetic foot ulcers consisted of fungal species. In this study, only one fungal genus, *Malassezia*, a skin commensal that can also be pathogenic (Findley et al., 2013; Ianiri et al., 2022; Vijaya Chandra et al., 2020), was detected in the DFU of certain participants. However, it is important to note that this study used saponin-based host depletion, which targets eukaryotic membranes to cause lysis. Saponins are known to have anti-fungal activity (Morrissey & Osbourn, 1999; Wittstock & Gershenzon, 2002), and hence the use of a saponin-based approach would not be suitable to study the mycobiome of DFUs. Additionally, the removal of reads aligned with the human genome prior to taxonomic classification also presents a bioinformatics challenge, since the similarities between microbial eukaryotic genomes and human genome could lead to removal of microbial eukaryotic reads as well. However, this does not mean that the DFU mycobiome has not been explored previously. Though fewer in number compared to studies that examine the bacteriome, previous studies have used molecular and culture-based techniques to identify members of the DFU mycobiome. Kalan et al. (2016) used Illumina sequencing of the internal transcribed spacer 1 region of eukaryotic rRNA to estimate that 80% of DFUs contain fungi, whereas culture-based methods identified only 5% of DFUs with positive yeast culture. The authors also reported that *Cladosporidium herbarum* and *Candida albicans* as the two most abundant species. The high prevalence of *Candida* in wounds has also been reported in various other studies (Chellan et al., 2010; Dowd et al., 2013). *Candida* also form polymicrobial biofilms with bacteria such as *Staphylococcus, Streptococcus* and *Pseudomonas*, and this can result in increased antimicrobial resistance (Harriott & Noverr, 2009; Kong et al., 2016; Lindsay & A., 2018; Short et al., 2023). Fungi have been linked to poor healing outcomes (Kalan et al., 2016), highlighting that though there is relatively less work on the DFU mycobiome, it has significant implications for wound healing.

The continuing rise of antibiotic resistance presents a severe concern for the treatment of wound infections. Resistant pathogens complicate healing processes, often resulting in chronic non-healing wounds and an increased risk of severe complications like sepsis or amputation. In a cohort study of DFUs, an *S. aureus* strain associated

with poor healing outcomes was observed to harbour multiple resistance genes (Kalan et al., 2019). The current study had a small sample size, and hence, it is likely that no statistical significance was observed for links between healing outcomes and antibiotic resistance genes. However, the identification of resistance genes corresponding to at least 12 drug classes in each participant of this study highlighted the high prevalence of AMR. Additionally, all DFUs showed the presence of resistance genes associated with penam, tetracycline and macrolide classes of antibiotics. This is alarming since the recommended first line of treatment for DFU infections is flucloxacillin (penam), followed by doxycycline (tetracycline), clarithromycin (macrolide) or erythromycin (macrolide) (National Institute for Health and Care Excellence - NICE guideline).

In this study, genes relevant to antibiotic efflux resistance mechanisms were detected in all participants, belonging to the major facilitator superfamily (MFS), ATP-binding cassette (ABC), multidrug and toxic compound extrusion (MATE) transporter and small multidrug resistance (SMR) efflux pump AMR families. One particularly interesting observation was the detection of *mex* genes in the DFU of Participant 6. These genes belong to the resistance-nodulation-cell division (RND) antibiotic efflux pump AMR gene family, important for multidrug resistance in Gram-negative bacteria. Particularly, *mex* genes have been associated with resistance in *P. aeruginosa* (Avakh et al., 2023; Ozer et al., 2012), a key pathogen identified in the microbiome of this participant. Surprisingly, *P. aeruginosa* was not reported as a causative pathogen in the clinical diagnosis, and thus, clinical information on its antibiotic resistance profile was lacking. However, the novel detection of this gene family in this participant's samples indicates a highly likely correlation with the presence of *P. aeruginosa*, which was not present within the top 10 species of any other participant. Efflux pumps play crucial roles in antibiotic resistance by actively expelling antibiotics from bacterial cells, thereby reducing intracellular drug concentrations and enabling bacterial survival. This is even more relevant in biofilms, where efflux pump genes are overexpressed (Alav et al., 2018). Recent studies have explored efflux pump inhibitors as novel antimicrobial therapeutics and shown that these inhibitors have the potential to reduce biofilm formation and resistance (Kvist et al., 2008; Reza et al., 2019).

The second resistance mechanism that was detected in all DFUs was antibiotic inactivation. Antibiotic inactivation by enzymatic mechanisms is a significant resistance strategy employed by bacteria, wherein specific enzymes chemically

modify or degrade antibiotics, rendering them ineffective. For example, β-lactamases hydrolyse the β-lactam ring of penicillins and cephalosporins, while aminoglycoside-modifying enzymes alter the structure of aminoglycosides (De Pascale & Wright, 2010). Kalan et al. (2019) also found genes conferring resistance to β-lactams to be highly prevalent in DFUs. In this study as well various β-lactamases (SHV, *blaZ*, OXA ACT, CARB-8, *cfxA*) were detected in all DFUs except for Participant 2 (which had the presence of an aminoglycoside nucleotidyltransferase gene). This highlights the presence of antibiotic inactivation mechanisms in the microbiome of wound infections, with various wound pathogens known to be β-lactamase producers and an increasing concern around the spread of ESBLs (Enoch et al., 2012; Livermore et al., 2015). In a study of infected DFUs, Mudrik-Zohar et al. (2022) found that patients who underwent amputation had a higher prevalence of β-lactamase producers.

Another key mechanism of resistance relevant to wound infections is resistance conferred by the *Staphylococcal* cassette chromosome *mec* (Lee et al., 2018). MSRA has been reported to be present in ~16% of diabetic foot infections (Stacey et al., 2019), and notably, the *mecA* gene in MRSA encoding for an altered penicillin-binding protein, PBP2a, has a low affinity for β-lactam antibiotics, including flucloxacillin (Nathwani et al., 2010). In this study, the *mecA* gene was not commonly detected, and it was present only in the DFUs of Participants 3 and 8.

While this study provides a novel perspective of DFU microbiomes using advanced sequencing technology, some limitations exist. This study highlights the difficulties in dealing with clinical samples and the effects of sample processing. The host depletion and DNA extraction method (saponin-based) was optimised using chronic wound samples (see Chapter 4). However, the samples used during optimisation originated from wounds on amputated limbs and thus represented wounds with extreme chronic conditions. The biomass obtained from such wounds was very high, whereas in the clinical study, only participants with grade 1 and 2 infections were recruited. Hence, the swabs obtained likely had a much lower biomass. Additionally, sampling technique has the potential to impact the detection of microbes (Gardner et al., 2006), since swab samples were collected by different members of the clinical team. This resulted in certain samples having non-detectable quantities of DNA. During library preparation, these samples underwent PCR-based barcoding with two additional PCR cycles than others and were still included in the analysis. Thus, the minor differences in sample

processing could impact sequencing outputs, though a negative control was used to normalise data across different sequencing runs.

A significant discrepancy was observed in the sequencing library sizes, even for samples processed within the same sequencing run. For instance, sample P3W1 yielded approximately 80,00,000 reads, whereas other samples within the same run had between 11,00,000 and 24,00,000 reads per sample. These large differences could be partly attributed to recent library preparation protocol updates with the new Nanopore chemistry kits. The latest 10.4.1 PromethION flow cells and the compatible kit (SQK-RBP114.24) require PCR products to be pooled before purification. Although advantageous when handling 24 samples, this method presumes that all samples will be purified in the same ratio as pooled, which might not always be the case. In the example of sample P3W1, the higher read count is likely due to the sample having a higher DNA concentration and purity than others, resulting in its disproportionate representation in the final library preparation.

Another limitation is the small sample size of this study, which reduces the statistical power of the analysis, making it difficult to generalise the findings to a broader population and potentially increasing the margin of error. The limited number of participants also makes identifying significant trends or patterns difficult. However, there are still advantages to this study despite the small sample size. Using Nanopore sequencing with the PromethION platform allows for high-resolution data that provides detailed insights into the microbial communities present in the wounds. The longitudinal design, monitoring changes over five weeks, adds significant value by offering a dynamic view of the microbiome in relation to treatment and healing outcomes. This approach can highlight temporal shifts and potential mixed community relationships. Even with a small cohort, this study provides valuable preliminary data and insights that can inform more extensive, more comprehensive future studies.

5.6 Conclusions

In this pilot study, temporal changes in the microbiome of diabetic foot ulcers (DFUs) were investigated using a small cohort. Key wound-relevant bacteria, including *Staphylococcus aureus, Corynebacterium striatum*, and *Finegoldia magna*, among others, were identified. Despite the limited sample size, the application of advanced Nanopore sequencing technology provided a comprehensive view of the DFU microbiome and its changes over time with treatment. The study underscored the limitations of culture-based diagnostics, revealing that species present in low abundance or those typically overlooked can be highly prevalent as DFU microbial dynamics change. This finding challenges the traditional focus on dominant pathogens and highlights the necessity of considering the entire microbial community. The identification of resistance genes, such as efflux pumps and β-lactamases, further emphasised the complexity of DFUs, with genes corresponding to multiple drug classes prevalent in each ulcer. While this study offers valuable insights, it also has limitations, including the small sample size and discrepancies in library sizes. These constraints suggest that larger cohort studies or longitudinal studies with more diverse populations are needed to validate and extend these findings. In conclusion, the insights gained from this study highlight the importance of evaluating the wound microbiome as a whole community when assessing wound infections and their treatment. The findings suggest that addressing the polymicrobial nature of DFUs and the presence of antibiotic-resistant bacteria requires a multifaceted approach. Integrating advanced molecular techniques with traditional culture-based diagnostics can enhance our understanding and inform more effective treatment and wound care strategies. By acknowledging the complexity of DFU microbiomes, future research and clinical practices can better address the challenges of chronic wound infections.

Chapter 6

Discussion and Conclusion

6.1 Discussion

The overarching aims of this thesis were to explore the dynamics of mixed bacterial communities associated with wound infections and evaluate methods for studying these communities in clinical contexts. By developing an *in vitro* mixed-bacterial community model and assessing the effects of antibiotics, this research aimed to gain insights into how these complex interactions influence treatment outcomes (Chapter 3). Furthermore, the evaluation and optimisation of host depletion and DNA extraction methods aimed to improve the analysis of wound microbiomes (Chapter 4), ultimately contributing to a more nuanced understanding of microbial dynamics in wound infections and their links to treatment and wound healing in a pilot study (Chapter 5). The results presented in this work provide a foundation for enhancing future microbiome research and highlight the importance of mixed microbial communities in wound infections.

Previous studies have shown that wounds are inhabited by various microorganisms, underscoring the presence of mixed-species communities in wounds (Giacometti et al., 2000; Johnson et al., 2018; Kalan et al., 2019; Liu et al., 2020; Loesche et al., 2017; Misic et al., 2014; Mohammed et al., 2017; Sloan et al., 2019; Tom et al., 2019; Tomic-Canic et al., 2020; Verbanic et al., 2020; Wolcott et al., 2016). Clinical diagnosis of wound infections largely relies on culture-based techniques, along with the use of automated systems such as VITEK (MALDI-TOF) for pathogen identification. Reporting in clinical microbiology diagnostic laboratories for diabetic wound infections focuses on identifying the most likely pathogen, often indicated by its dominant presence in culture. While this is useful, it overlooks the wound's diverse microbial community. The presence of these numerous microbial members of the wound community could potentially influence antibiotic treatment outcomes and thus affect wound healing. The formation of biofilms in wounds further exacerbates the problem, increasing their recalcitrance to antibiotics. Indeed, previous studies have explored the role of multi-species biofilms using numerous *in vitro, in vivo* and *ex vivo* models (Brackman & Coenye, 2016; Cardenas-Calderon et al., 2022; Dhekane et al., 2022; Hill et al., 2010; Jensen et al., 2017; Vyas et al., 2022). The aim of Chapter 3 was to explore the dynamics of multi-species communities in response to antibiotic exposure using a simple in *vitro* model. This static, rapid and reproducible model revealed a

protective effect of mixed communities, wherein a resistant strain could provide protection against antibiotics to other sensitive strains of the community. A key strength of this study was that the model included three relevant species and was evaluated in both planktonic and biofilm lifestyles, highlighting the differences between them. Additionally, the study tested the effect of mixed-microbial cultures upon antibiotic exposure to three different antibiotics – two β-lactams and one bacteriostatic tetracycline antibiotic. The protective effect was not just limited to penicillin but also seen for flucloxacillin, the first line of treatment for diabetic foot infections where Gram-positive organisms are identified as the causative pathogens. This is alarming since it indicates that, in clinical settings where only predominant organisms are identified and flucloxacillin could be prescribed, the presence of other resistant strains in the microbial community of the wound could potentially protect the causative pathogens.

Having identified the importance of mixed microbial communities *in vitro* and how a mixed community serves as a factor for protection against antibiotics, the study aimed to identify microbial dynamics of wound microbiota in the clinical context. Previous studies of the human microbiome have highlighted the issues around high levels of contamination associated with host (human) DNA (Gevers et al., 2012; Marotz et al., 2018), resulting in a 'waste' of sequencing power and reduced understanding of the microbial components of the samples. Samples obtained from wounds are known to have high levels of human DNA, owing to the presence of wound fluid, tissue debris and various host-derived factors. To address this, in Chapter 4, different pre-sequencing host depletion methods were evaluated for their effectiveness in reducing human DNA and their impact on microbial DNA. The findings of this study suggest that host depletion using 5% saponin and DNAse treatment was the most effective in reducing human reads, enriching bacterial reads and detecting more low-abundance species. This is supported by the successful application of this 5% saponin-based method to respiratory microbiome samples (Charalampous et al., 2019; Yang et al., 2019). Importantly, this chapter also emphasises the need to refine host depletion techniques in the context of specific sample types. This is supported by previous work, where studies that have evaluated different commercial kits and other host depletion approaches have obtained highly variable results, all influenced by sample types, sample processing and technique of quantification, amongst other factors (Bruggeling et al., 2021; Hasan et al., 2016; Klosinska et al., 2022; Marchukov et al., 2023; Marotz

et al., 2018; Nelson et al., 2019; Shi et al., 2022; Street et al., 2019). Based on available literature to date, this research represents the first evaluation of multiple pre-sequencing host depletion techniques using advanced long-read Nanopore sequencing technology in wound microbiome studies.

Alongside comparing different host depletion methods, the research investigated the use of an optimal bioinformatics pipeline for data analysis. While cloud-based tools like Oxford Nanopore Technologies' EPI2ME What's In My Pot (WIMP) workflow exist, an in-house workflow (Freiheit, 2023) was found to be more advantageous. This workflow used Kraken2 for taxonomic classification, in comparison to Centrifuge used by WIMP. While a key benefit of Centrifuge is its reduced memory usage, this was not a limitation given the access to the University of Hull's high-performance computing facilities. Additionally, Kraken2 does not allow multiple taxonomic assignments to a single read unlike Centrifuge. Allnutt et al. (2021) demonstrated that Kraken2 had better recall and precision than Centrifuge regarding bacteria, viruses and eukaryote taxonomic classification. An in-house workflow also allowed for tailored adjustments specific to the project's needs, such as data processing steps, taxonomic classification methods, and the building of a customised database. For instance, Freiheit (2023) incorporated contig assembly into the updated workflow, addressing the evolving bioinformatics needs of the project (Chapter 5).

The use of the selected host depletion method also addresses, in part, a unique challenge associated with sequencing technologies. While sequencing technologies provide novel insights, it is difficult to assess the viability of detected species, since the DNA being sequenced could originate from live, dead or extracellular sources. The use of saponin and a DNAse treatment step increases the likelihood that extracellular DNA is degraded (Nelson et al., 2019).

After identifying a host depletion and DNA extraction method suitable for the sample type, it was subsequently applied to clinical samples. The clinical study aimed to examine microbiome dynamics and identify changes over time and with treatment (Chapter 5). A key advantage of this study was not only the application of advanced long-read sequencing to understand the wound microbiome, but also the ability to link into traditional culture-based microbiology diagnosis and clinical outcomes. After sequencing was performed for these clinical samples, a difference in library size was

noted for each, with the number of reads varying greatly. This was, in part, attributed to the low yield from some clinical samples, as well as the differences in library preparation protocol, which could have yielded unequal amounts of each sample in the pooled library prior to sequencing. Hence, a decision was made to rarefy sequencing data prior to any analysis. There is significant debate around rarefaction since it can lead to data loss (Weiss et al., 2017). However, when there is a considerable discrepancy between the smallest and largest library sizes, rarefaction can help ensure that analyses are not dominated by a few samples with disproportionately high read counts. This can be particularly important for metrics that are sensitive to library size, like diversity indices.

Despite a small cohort size (n = 9), comprehensive insights into the microbiome were provided in Chapter 5. This research explored the plausible links between microbiome dynamics, wound size, clinical pathogen diagnosis and antibiotic treatments. The results show critical discrepancies between traditional culture-based diagnostics and molecular sequencing approaches in accurately identifying the microbial composition of wound infections. Notably, sequencing data revealed a significant enrichment of *Finegoldia magna* in non-healing wounds - a Gram-positive anaerobe that was not reported in culture-based diagnostics of the cohort, except in one participant where an anaerobe was identified. Still, no further information on genus or species was reported. The clinical relevance of *F. magna* is significant, as it has been increasingly associated with diabetic wound infections and has various virulence factors that may play a role in delayed healing (Citron et al., 2007; Dowd et al., 2008b; Jneid et al., 2018; Murphy et al., 2014; Smith et al., 2016; Villa et al., 2024; Wolcott et al., 2016). The underreporting of *F. magna* in culture-based methods raises concerns about the potential for incomplete diagnosis and suboptimal treatment, particularly in non-healing wounds where accurate identification of pathogenic species is crucial. Moreover, the study underscored the presence of *Corynebacterium striatum*, a Gram-positive organism consistently identified by sequencing but not reported in culture diagnosis. *C. striatum* is often dismissed as a contaminant or part of the normal skin flora in culture-based diagnostics (Scharschmidt & Fischbach, 2013; Soriano & Zuckerman, 2018; Streifel et al., 2022). This can lead to its underreporting in culture-based diagnostics. Additionally, in polymicrobial samples, *C. striatum* may be overshadowed by more rapidly growing or dominant organisms preferentially identified

in culture. However, its frequent detection through sequencing (Johani et al., 2019; Kalan et al., 2019; van Asten et al., 2016) suggests it may have a more active role in wound pathology than previously recognised.

These findings support the need for more integrative diagnostics, possibly using a dual approach of culture-based methods and advanced molecular techniques. Such an approach would provide a more comprehensive understanding of the wound microbiome, particularly in chronic or non-healing wounds where identifying all relevant microbial species is critical for guiding effective treatment strategies. This point is reinforced by the findings in Chapter 3, which demonstrated a clear protective effect against antibiotics by certain members of the microbial community. Therefore, in a clinical setting, failing to identify members of the wound microbiota could result in overlooking the protective roles that some microbes, not traditionally classified as causative pathogens in culture-based diagnosis, may play. Chapter 5 also revealed that antibiotic resistance was prevalent across all diabetic foot ulcers, with resistance genes related to multiple drug classes detected in every participant. The consistent presence of tetracycline, penam, and macrolide resistance genes, which correspond to commonly used treatments, is particularly concerning.

6.1.1 Study limitations

While this research provides insights into mixed microbial communities associated with wounds, several limitations should be considered. Chapter 3 indicated a protective effect in mixed-microbial communities *in vitro*. Alongside the limitations of this study stated in Chapter 3 (section 3.5), it is important to highlight that real wounds are dynamic environments with varying oxygen levels, pH, and nutrient availability (Guo & Dipietro, 2010), which can influence microbial behaviour and interactions in ways that may not be fully replicated in simple static *in vitro* models. The model used in this work also does not consider host factors such as host skin cells, immune responses, tissue interactions, or the presence of host-derived antimicrobial peptides, which play an essential role in wound healing (Herman & Herman, 2019; MacLeod & Mansbridge, 2016; Pfalzgraff et al., 2018). These factors can significantly influence microbial survival and antibiotic resistance in wounds, affecting how microbes interact

and respond to treatment. The *in vitro* model developed herein does consider biofilm formation. However, *in vivo,* biofilm structures can differ, where physical factors like tissue structures and fluid flow influence them. Additionally, to create a more controlled experiment setup, a three-species model was utilised. In reality, however, the wound microbiota is likely to have many more members of the community. Thus, the *in vitro* model may not fully reflect the complexity of microbial communities in real wounds. Further, the effects of antibiotics on mixed communities in a living host can be influenced by additional factors such as drug distribution and metabolism.

There are also differences in the sequencing technology used during the optimisation of host depletion methods in Chapter 4, and their application to clinical samples in Chapter 5. At the time of executing sequencing, as outlined in Chapter 4, the sequencing facility (University of York Genomics Services) had access to the MinION platform. Hence, during host depletion optimisation, the study used 9.4.1 MinION flow cells and the Rapid PCR Barcoding Kit (SQK-RBP004), where each sample was PCR-barcode individually, purified with Ampure XP Beads, and then pooled in equimolar ratios. However, during the execution of sequencing in Chapter 5, the manufacturer discontinued the previously used kits and flow cells. Considering further availability of resources and financial constraints, using the PromethION sequencing platform was considered the best option. Flow cells of R10 chemistry (FLO-PRO114M), and the Rapid PCR Barcoding Kit V14 (SQK-RBP114.24) with 24 barcodes per run were used. Although the transition from R9 to R10 chemistry reflects advancements in pore design and overall sequencing quality (Ni et al., 2023), variations in compatible kits between these versions led to changes in the library preparation method. In contrast to the previous version, The Rapid PCR Barcoding Kit V14 required PCR products to be pooled before purification. This change in workflow, while efficient for processing 24 samples, introduces the risk that the pooling ratio may not be perfectly uniform. This could potentially result in a cleaned pooled sample that does not have the same ratio as the initial pooling, thus affecting the loaded library and eventually affecting the results. These variations in the sequencing kits and library preparation methods highlight the challenges in maintaining methodological consistency across constantly updating sequencing technologies and contribute as a study limitation.

Another key limitation of the study of the wound microbiome outlined in Chapter 5 was the limited sample size. Although the study was designed with the goal of recruiting 24 participants—based on the clinic's estimation regarding patient frequency and to ensure a sufficient sample size for statistical analysis—only 9 participants were recruited by the end of the sample collection period. This reflects the challenges of managing the study's clinical and laboratory aspects. Balancing clinic attendance to evaluate the suitability of potential participants, conduct recruitment, and complete consent procedures alongside longitudinal, laboratory-based research presented logistical difficulties. Although efforts were made to increase clinic presence during key periods, this did not significantly enhance recruitment rates. Practical issues during implementation also arose; for instance, patient visits were subject to various factors such as clinical needs and scheduling constraints, and wound swabs for microbiology diagnosis were not always collected as anticipated, resulting in a smaller cohort size. However, the longitudinal aspect of the study allowed for a deeper analysis of each participant's microbiome dynamics over time, providing valuable insights despite the limited sample size. This approach provided a detailed, case-study-like examination of individual ulcer infections rather than merely focusing on broader cohort-wide features such as predominant microorganisms, diversity indices, and general distinctions between healing and non-healing wounds. Despite the small cohort size, interacting with the participants and clinical team demonstrated the clinical relevance and translational impact of the research undertaken in this thesis.

Another limitation of this study was that it primarily focused on the role of bacteria in mixed-species communities in wound infections. The *in vitro* model in this work incorporated only bacterial species, and while fungi were not explicitly excluded from the analysis of the clinical wound microbiome, very few fungi were detected. This is likely due to the host depletion method which uses saponin, known to have anti-fungal activity (Morrissey & Osbourn, 1999; Wittstock & Gershenzon, 2002). Hence, it is likely that there was loss of fungal DNA during the DNA extraction process. It is important to acknowledge that fungi should not be overlooked. Fungi such as *Candida spp.* form an important part of the wound microbiota (Kalan et al., 2016; Lindsay & A., 2018; Short et al., 2023) and their presence has been associated with poor prognosis and healing outcomes. Despite this, there are difficulties in characterising the wound mycobiome. Similar to the use of 16S rRNA gene sequencing for bacterial

identification, the internal transcribed spacer (ITS) region is used for fungal identification (Nilsson et al., 2019) through PCR-based amplification and DNA sequencing. However, the ITS region does not always provide enough resolution and short-read sequencing technologies often struggle to accurately differentiate closely related fungal species (Hoang et al., 2019; Lucking et al., 2020). In contrast to short-read sequencing technologies, long-read sequencing platforms such as Oxford Nanopore Technologies offer improved resolution of fungal genomes, providing a potential solution to some of these challenges. However, these platforms still face significant hurdles. For instance, despite using both ITS and large subunit (LSU) regions as the target for PCR amplification and Nanopore sequencing, Ohta et al. (2023) found that it failed to provide species-level resolution for specific fungi. Additionally, when applied to sputum samples, a large amount of contaminant human DNA was also present. More refined host depletion techniques are needed to remove human DNA but preserve fungal DNA. Due to the rigid and resilient nature of the fungal cell wall, the choice of DNA extraction methods can also impact the ability to study the mycobiome (Fredricks et al., 2005). Furthermore, databases for fungal classification require improvement to enable accurate taxonomic assignment (Halwachs et al., 2017; Nilsson et al., 2019). Kalan et al. (2016) found that 32% of diabetic foot ulcer samples in their study contained fungal sequences that could not be classified beyond the kingdom level, highlighting the limitations of current fungal reference databases.

6.1.2 Future work

Several avenues for future research are suggested to build upon the findings of this study and address its limitations. Considering the *in vitro* model developed in this study and its findings, future research can incorporate more complex, human-relevant models, such as *ex vivo* tissue cultures or animal models, which better mimic the conditions of actual wound infections and improve the translational value of the findings. Additionally, it would be interesting to evaluate the dynamics of mixed communities upon antibiotic exposure when different microbial members, such as skin commensals, fungi, and a mix of aerobes and anaerobes, are considered. Drawing on the findings of Chapter 5, it would be insightful to see the effects of including an *F. magna* or *C. striatum* strain in the mixed community model. It would also be important

to consider if the observed protective effects are sustained over longer periods, mimicking the duration of antibiotic treatment in the clinic.

To validate and further support the findings of Chapter 4, the inclusion of an increased range of samples (in terms of infection grade and chronic status) in evaluating host depletion methods can be useful. Additionally, to study the mycobiome of wounds, it would be important to test the performance of different host depletion methods with respect to fungal DNA.

The findings from Chapter 5 indicated that certain wound microbiome members are likely overlooked in the clinical context. The study prompts further investigation into the pathogenic potential of organisms like *C. striatum*, which may be underestimated in clinical settings, and the broader implications of overlooking anaerobic bacteria such as *F. magna*. Future research should focus on refining diagnostic methods to improve the detection and characterisation of wound-associated microbiota, ultimately enhancing patient outcomes by facilitating more targeted and effective therapies.

Additionally, to address the key limitation of small sample size, future work can focus on larger sample numbers to enhance the statistical power and generalisability of the findings. Improved study designs that can incorporate increased recruitment, multiple recruitment centres, and extended follow-up periods can enrich the data obtained from such studies. Increased sample numbers will also reflect inherent variability within such diabetic foot ulcers.

While the use of saponin and DNAse reduces the likelihood of sequencing extracellular DNA, there is still difficulty in differentiating between live and dead microbial cells. Future studies can also incorporate the use of PMA (Propidium Monoazide) technique for non-viable cell exclusion. The PMA technique has been combined with sequencing approaches before, though its performance in real-world complex microbial communities has not been as optimal (Wang et al., 2021a).

Studies that implement long-read Nanopore sequencing with platforms such as the PromethION provide a significantly large amount of data. While this is beyond the scope of this PhD, there is great potential in extracting more information from that data, such as interaction networks or the identification and annotation of genes, including those associated with specific virulence factors, toxins, and metabolic pathways.

Future studies can also combine DNA sequencing with transcriptomics of host tissue, thus improving our understanding of links between host factors and the wound microbiome. Tipton et al. (2020) highlighted this potential, demonstrating that host genetic factors influencing cellular adhesion can significantly impact wound microbiome diversity and infection outcomes.

Finally, like previous studies, this work highlights the potential of molecular-based approaches in routine microbiology diagnosis in the clinic, overcoming the limitations of culture-based techniques. However, there are currently various barriers to being able to apply these advanced molecular methods, including small-scale studies, viability, antimicrobial phenotypes, access to equipment and associated costs (Sande et al., 2023). Addressing these concerns is crucial to ensure the accuracy, reliability, and clinical relevance of these techniques before their application in the clinic. Rather than serving as a replacement for traditional culture-based identification and antimicrobial susceptibility testing, advanced molecular methods should be viewed as complementary tools that enhance diagnostic precision and effective treatment. While culture-based methods are currently essential for determining viable pathogens and guiding treatment decisions, molecular techniques can provide a broader and more rapid understanding of the microbial landscape, including the detection of difficult-to-culture microorganisms, and can identify antimicrobial resistance genes. Integrating both approaches could lead to a more comprehensive diagnostic strategy eventually improving patient outcomes.

6.2 Conclusions

This research aimed to address a critical gap in understanding the dynamics of mixed bacterial communities in wound infections. The multi-faceted approach used in this thesis aimed to (1) build an *in vitro* mixed-bacterial community model and evaluate the effects of antibiotics on population dynamics; (2) test a range of host depletion and DNA extraction methods for their suitability for wound samples; and (3) apply the optimised host depletion method to clinical samples to understand the dynamics of the wound microbiome.

All three aims of the research were successfully addressed, and novel insights and findings from these are presented in this thesis (Figure 6.1). Overall, this research developed *an in vitro* model incorporating both planktonic and biofilm states and supported the growth of three wound-relevant bacterial species in mixed communities. The model revealed that mixed communities can provide a protective effect to their sensitive members against different antibiotics. Next, five different host depletion and DNA extraction methods were evaluated to enable the study of microbial communities in wounds. From these, a 5% saponin-based method was found to be the optimal choice for wound-relevant samples. This method was then applied to clinical samples, identifying changes in the wound microbiome with time and treatment and in the context of clinical diagnosis of infection and wound healing state.

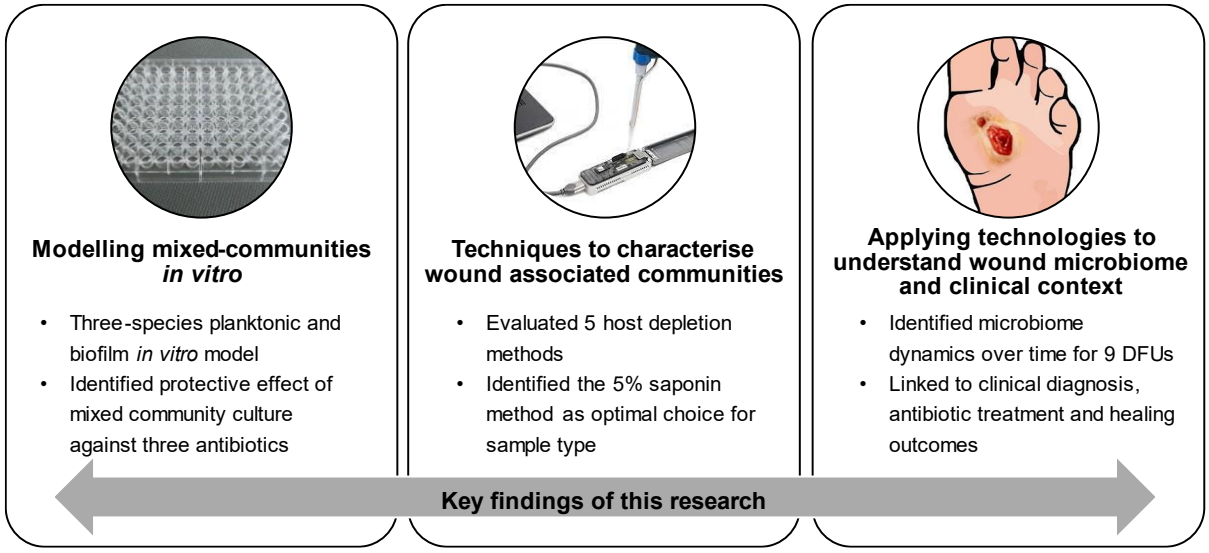

Figure 6.1: The multi-faceted approach used in this research and their key findings.

By continuing to bridge the gap between laboratory research and its clinical context, we can better understand the complex nature of wound microbial communities. In conclusion, the insights gained through this research represent a meaningful contribution to our understanding of mixed microbial communities in wounds, with the potential to inform and impact clinical diagnosis and treatment in the future.

Chapter 7

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Chapter 8

Appendix

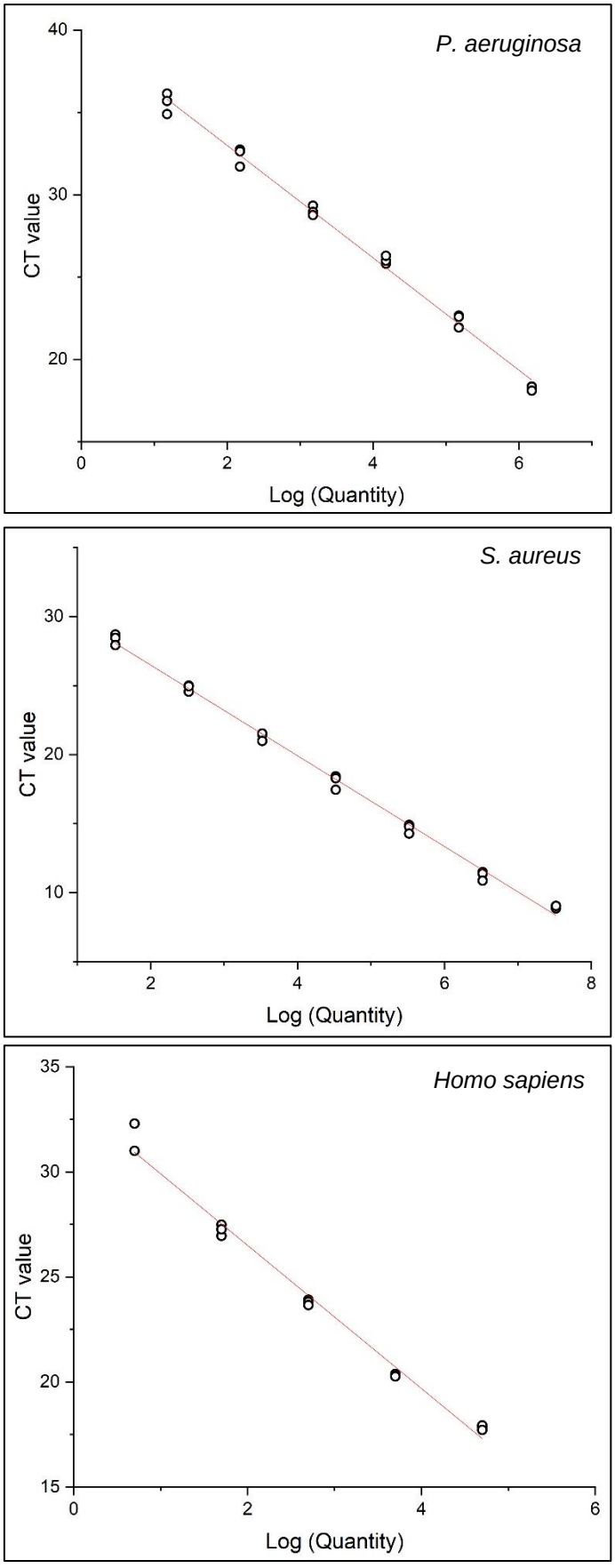

Figure A1: qPCR Standard Curve for P. aeruginosa, S. aureus and H. sapiens.