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The Microbiome of Diabetic Foot Ulcers and the Role of Biofilms

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

By

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ABSTRACT

Diabetic Foot Ulcers are a common precursor to the development of infection and amputations. A breach in the protective skin barrier represents a portal of entry for invading microorganisms, where infective episodes frequently pursue. Three key areas that may augment clinical care are one. understanding what microorganisms are present in Diabetic Foot Ulcers, two. differentiating if microorganisms are planktonic microbial cells or slow growing microbial biofilms and three. treating Diabetic Foot Ulcers complicated by microorganisms with effective topical agents.

As part of this thesis, 16S rDNA next generation sequencing was utilised to profile the microbiota of infected Diabetic Foot Ulcers (DFUs). Clinical / laboratory data and treatment outcomes were collected and correlated against microbiota data. Thirty-nine patients with infected DFUs were recruited over twelve-months. Shorter duration DFUs (<six weeks) all had one dominant bacterial species (n= five of five, 100%, p <·001), *S. aureus* in three cases and *S. agalactiae* in two. Longer duration DFUs (≥six weeks) were diversely polymicrobial (p = .01) with an average of 63 (range 19-125) bacterial species. Severe Diabetic Foot Infections (DFIs) had complex microbiota's and were distinctly dissimilar to less severe infections (p = .02), characterised by the presence of low frequency microorganisms.

Our results confirm that short DFUs have a simpler microbiota's consisting of pyogenic cocci but chronic DFUs have a highly polymicrobial microbiota. The duration of a DFU may be useful as a guide to directing antimicrobial therapy.

Secondly, we utilised Scanning electron microscopy (SEM) and Fluorescent in situ Hybridisation (FISH) techniques to determine if DFUs were complicated by sessile, slow growing bacteria referred to as biofilms. 65 DFU specimens were obtained from subjects with infected chronic ulcers. Of the 65 DFU specimens evaluated by microscopy, all were characterized as containing biofilm (100%, $p < .001$). Molecular analyses of DFU specimens revealed diverse polymicrobial communities. No clinical visual cues were identified in aiding clinicians identify wound biofilm. Microscopy visualization when combined with molecular approaches, confirms biofilms are ubiquitous in DFUs and a paradigm shift of managing these complicated wounds needs to consider anti-biofilm strategies.

Lastly, the effectiveness of various topical antimicrobials commonly used in woundcare were tested in two separate studies by employing *in vitro* models, *ex vivo* porcine skin explant models and *in vivo* human studies. In the first study, 17 participants with chronic non-healing DFUs due to suspected biofilm involvement were recruited to receive one-week application of Cadexomer Iodine ointment. Real-time qPCR was used to determine the microbial load with 11 participants exhibiting one-two Log10 reductions in microbial load after treatment, in comparison to six patients who experienced <one log10 reduction (p =.04). Scanning electron microscopy (SEM) and/or fluorescent in situ hybridisation (FISH) confirmed the presence or absence of biofilm in all 17 participants. 16S rDNA next generation sequencing provided useful insights that these wounds support complex polymicrobial communities and demonstrated that Cadexomer Iodine had a broad level of antimicrobial activity in reducing both facultative

anaerobes such as *Staphylococcus* spp., *Serratia* spp., aerobes including *Pseudomonas* spp., and obligate anaerobes including *Clostridiales family XI*.

In the second study, a range of topical antimicrobial wound solutions were tested under three different conditions; (*in vitro*) 4 % w/v melaleuca oil, polyhexamethylene biguanide, chlorhexidine, povidone iodine and hypochlorous acid were tested at short duration exposure times for 15-minutes against three-day mature biofilms of *S. aureus* and *P. aeruginosa*. (*ex vivo*) Hypochlorous acid was tested in a porcine skin explant model with twelve cycles of ten-minute exposure, over 24 hours, against three-day mature *P. aeruginosa* biofilms. (*in vivo*) 4 % w/v Melaleuca Oil was applied for 15-minutes exposure, daily, for seven days, in ten patients with chronic non-healing Diabetic Foot Ulcers (DFUs) complicated by biofilm.

In vitro assessment demonstrated variable efficacy in reducing biofilms ranging between 0.5 log10 reductions to full eradication. Repeated instillation of hypochlorous acid in a porcine model achieved < one log10 reduction (0.77 log10, $p < 0.1$). Application of 4 % w/v melaleuca oil *in vivo*, resulted in no change to the total microbial load of DFUs complicated by biofilm (median log10 microbial load pre-treatment = 4.9 log10 versus 4.8 log10 ($p = .43$).

In conclusion, to the best of our knowledge, the *in vivo* human studies testing the performances of topical antimicrobials represents the first *in vivo* evidence employing a range of molecular and microscopy techniques. These demonstrate the ability of Cadexomer Iodine (sustained release over 48-72 hours) to reduce the microbial load of chronic non-healing DFUs complicated by biofilm. In contrast, short durations of exposure to topical antimicrobial wound solutions commonly utilised by clinicians are ineffective against microbial biofilms, particularly when used *in vivo*.

DEDICATION

I would like to dedicate this thesis to my family. Undertaking a thesis is a giant commitment but doing this while working as a clinician within the healthcare system is beyond extraordinary. Many hours sitting in front of a computer are required, and striking a fine balance between work, your thesis and family life is challenging. To do this you need the love and support of your family. To my wife Victoria, and my sons Theodore and Huey, who through all the hard times and late nights provided me with the strength to prevail, thank you for being there for me. Without this continual support and love, finishing this thesis would not have been possible.

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CONTRIBUTORS

I would like to express my gratitude and thanks to my collaborators who contributed to this thesis by performing laboratory-based experiments or procedures that contributed to my work.

For chapters two-six, I acknowledge the fee-paying facilities, scientific and technical assistance of the Australia Centre for Ecogenomics, University of Queensland who performed the DNA sequencing of tissue samples.

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For chapter two section 2.2, I was invited by the Global Wound Biofilm Expert Panel to be the senior/primary author on a position document and meta-analysis and systematic review for wound biofilm. I declare the manuscripts to be undertaken primarily by myself using literature contained within chapters one and two, and in agreement of the co-authors that the material would be included as part of my PhD thesis introduction/literature review.

For chapter five, I would like to acknowledge Associate Professor Sue McLennan of the University of Sydney for collaboration on the project. A/Prof McLennan performed gel band zymography to depict protease levels in wounds pre-and-post treatment with the topical antimicrobial, Cadexomer Iodine.

For chapter six, I would like to acknowledge Professor Gregory Schultz and Dr Qin Yang of the University of Florida. This collaboration saw the amalgamation of data produced from this thesis (*in vitro* and *in vivo*) in addition to the *ex vivo* porcine skin explant model performed by Professor Schultz and Dr Qin Yang. This collaboration for antimicrobial wound wash treatments strengthened our work significantly.

For chapter seven, I would like to acknowledge Professor Paul Stoodley and Dr Darla Goeres for their invaluable input into this review article.

STATEMENT OF ORIGINAL AUTHORSHIP

This thesis contains no material which has been accepted for the award of any other degree or qualification at any other University. To the best of my knowledge and belief, this thesis is original and contains no material previously published or written by another person, except where due reference has been given in the text.

Signed 

Date: 10th September 2018

(Matthew Malone)

LIST OF PUBLICATIONS BY CANDIDATE

Book Chapters:

Malone, M. (2017) The Microbiome of Diabetic Foot Ulcers and the Role of Biofilms In Kon, K., & Rai, M., *The Microbiology of Skin, Soft Tissue, Bone and Joint Infections* (pp. 41-56). London, UK, Academic Press, Elsevier.

Journal publications:

Johani, K., **Malone, M.,** Jensen, S.O., Gosbell, I. B., Dickson, H. G., Hu, H. and Vickery, K. Microscopy visualisation confirms multi-species biofilms are ubiquitous in diabetic foot ulcers. *International Wound Journal* 2017. doi:10.1111/iwj.12777.

Malone, M., Johani, K., Jensen, S. O., Gosbell, I. B., Dickson, H. G., Hu, H., Vickery K. Next Generation DNA Sequencing of Tissues from Infected Diabetic Foot Ulcers. *EBioMedicine* 2017. http://dx.doi.org/10.1016/j.ebiom.2017.06.026.

Johani, K., **Malone, M.,** Jensen, S. O., Gosbell, I. B., Dickson, H. G., Hu, H., Vickery K. Evaluation of short exposure times of antimicrobial wound solutions against microbial biofilms: From *in vitro* to *in vivo*. *J Antimicrob Chemother*. Accepted 18th November 2017, https://doi.org/10.1093/jac/dkx391.

Malone, M., Johani, K., Jensen, S. O., Gosbell, I. B., Dickson, H. G., McLennan, S., Hu, H., Vickery K. Effect of cadexomer iodine on the microbial load and diversity of chronic non-healing diabetic foot ulcers complicated by biofilm *in vivo*. *J Antimicrob Chemother* 2017; 72 (7): 2093-2101. doi: 10.1093/jac/dkx099.

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Malone, M., Goeres, D., Gosbell, I.B., Jensen, S.O., Vickery, K., Stoodley, P. Approaches to biofilm-associated infections: the need for standardized and relevant biofilm methods for clinical applications. *Expert Review Anti-Infective Therapy* 2016. Feb;15(2):147-156. http://dx.doi.org/10.1080/14787210.2017.1262257.

Letters:

Malone, M., Dickson H. Understanding the role of Fungi in Chronic Wounds. *MBio* 2016. (7), 6; e01898.

LIST OF CONFERENCE PRESENTATIONS BY CANDIDATE

- **Biofilms in wounds and bone.** Invited Speaker

✓ 5th National Diabetic Foot Infection Symposium, Turkish Clinical Microbiology and Infectious Diseases (KLIMIK) Association, Selcuk-Izmir, Turkey, May 2018.

- **The Microbiome of Diabetic Foot Infections: Do we need to change clinical practice?** Invited Speaker

✓ Diabetic Foot Australia bi-annual conference, Gold Coast, Australia, September 2017.

- **Biofilms and their role in chronic wounds.** Invited Speaker

✓ European Wound Management Association **(**EWMA) Annual Conference, Amsterdam, Netherlands, May 2017.

- **The Microbiome of Diabetic Foot Infections: Do we need to change clinical practice?** Invited Speaker

✓ DFCon, Texas, United States, March 2017.

- **Microbiome and the role of biofilms in Diabetic Foot Ulcers.** Invited Speaker

✓ World Union Wound Healing Societies (WUWHS) annual bi-annual conference, Florence, Italy, September 2016

- **Microbiome of Diabetic Foot Infections.** Oral presentation

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LIST OF ABBREVIATIONS

agr - Accessory gene regulator

AHL - Acyl-homoserine lactones

AIP - Autoinducing peptides

AMPs - Antimicrobial peptides

bTEFAP - Bacterial tag-encoded FLX amplicon pyrosequencing

cfu - Colony forming units

CLSM - Confocal laser scanning microscopy

CRP - C Reactive protein

DGGE - Density gel electrophoresis

DFU/s - Diabetic Foot Ulcer/s

DFI/s - Diabetic Foot Infection/s

DNA - Deoxyribose nucleic acid

eGFR - Estimated glomerular filtration rate

EPS - Extracellular polymeric substance

ESR - Erythrocyte sedimentation rate

FRACS - Full ribosomal amplification, cloning and Sanger sequencing

Gb – Gigabyte

HbA1c - Glycosylated haemoglobin

hBD - β-defensins

IDSA - Infectious Disease Society of America

KC - keratinocytes

MMP - Matrix metalloproteinases

MPS - Massively parallel sequencing

MRSA - Methicillin resistant *S. aureus*

MSSA - Methicillin sensitive *S. aureus*

NGS - Next generation sequencing

NF-kB-Nuclear factor kappa B

NPWT - Negative pressure wound therapy

SEM - Scanning electron microscopy

SI - Silhouette score

NPWTi - Negative pressure wound therapy and instillation

OTUs - Operational taxonomic units

PCR - Polymerase chain reaction

PHMB - Polyhexamethylene biguanide

PNA-FISH - Peptide nucleic acid fluorescent in situ hybridisation

PRAPS - Partial ribosomal amplification and pyrosequencing

PI/s - Pressure injuries

PRR - Pattern recognition receptors

PIA - Polysaccharide intercellular adhesion

PVP-I- Povidone iodine

PAMPs- Pathogen-associated molecular patterns

PU/s - Pressure ulcers

PCoA – Principal coordinates analysis

spp - Species within a genus

TEM - Transmission electron microscopy

TLR - toll like receptors

VLU/s - Venous leg ulcer/s

μm - /Micrometer

WGS - Whole genome shotgun sequencing

WCC - White cell count

PERMANOVA – Permutational multivariate analysis of variance

QS - Quorum sensing

rRNA - Ribosomal ribonucleic acid

SETTING THE SCENE

Diabetic foot ulceration (DFU) is a significant complication of diabetes and is associated with increased morbidity, mortality and increased health care expenditure. There are many facets to diabetic foot disease that contribute to the development of a DFU, in particular the "triad" of factors - peripheral neuropathy, peripheral vascular disease and trauma [1]. The pathway to amputation following a foot ulcer is a complex array of intertwining pathways with infection playing a key role. In a person with diabetes, foot ulceration leaves a physical break in the protective barrier of the skin. Invading microorganisms may colonize the wound, in which a impaired immune response is common in a person with diabetes. This may predispose an ulceration to further microbial invasion and replication, resulting in damage to host tissues and an inflammatory response that is characterized as clinical infection [2]. In a person with diabetes over 90% of infections are the primary pathway to lower extremity amputation, thus the significance of developing a foot infection in a person with diabetes is evident [3].

In a significant proportion of people with diabetic foot infection (DFI) the severity is enough to cause hospitalisation. Recent data by Malone and colleagues (2014) [4] indicated that infection was the primary cause of admission in 82% of patients with diabetes on a vascular surgery ward. It is perhaps not surprising then that the economic burden of managing infection in people with diabetes has been reported at an average of $17,879 per hospital separation [5].

The importance of managing DFI is underpinned by the requirement to identify the pathogen so as to direct antimicrobial therapy. Traditionally, wound cultures have been utilised to

identify planktonic pathogenic microorganisms, in addition to looking at their density through qualitative and quantitative measures [6]. The limitation of this method is that culture-dependent techniques select for species that flourish under the typical conditions of the diagnostic microbiology laboratory, and this may not necessarily reflect the most abundant or clinically important microorganisms in DFI [7]. Molecular DNA-based techniques that are culture-independent have identified the limitations of traditional cultivation-based methods when examining the wound microbiota. Amplification and sequence analysis of 16S rDNA, a highly-conserved gene present in all prokaryotes (bacteria) but not eukaryotes (humans), has revealed a vastly more complex array of bacterial communities in chronic wounds [8-10].

Additionally, research into the role of microorganisms in causing DFI has identified contrasting evidence suggesting that changes in management paradigms are required [11]. The roles of single free-floating microorganisms (planktonic) that are responsible for acute infections and readily identified through cultivation-based approaches differ vastly from multi-species sessile microorganisms typically not found by the same cultivation methods. The role of these sessile microbes and their significance in causing persistent infections in chronic wounds has only recently been appreciated [8]. Data from scanning electron microscopy of biopsies from chronic wounds have identified that 60% to 90% of specimens contained biofilm structures, in comparison to only 6% of biopsies from acute wounds [12-13].

Overall, limited evidence is available from both molecular and microscopy techniques to identify which microorganisms might be of importance in infected DFUs. A vast diversity of microorganisms from clinically uninfected DFUs has been found, extending the view of the diabetic foot microbiota. There remains, however, a large gap in knowledge regarding the specific roles of all of the microorganisms detected in infected DFUs and their relevance to

clinical care. Importantly, few studies have attempted to explore the relationship between infected DFUs and the presence of biofilm phenotypes through combinations of molecular and microscopy methods.

“Gentleman: It is the microbes who will have the last word”

- Louis Pasteur

THESIS OVERVIEW

Chapters 1 and 2 of this thesis provides an overview of the literature, highlighting the contributions of diabetes as a comorbidity and how it converges to create unique circumstances that increase the risk of infection and amputation. The bacteriology of DFI through culture-dependent techniques heavily implicate aerobic Gram-positive cocci as the predominant pathogens of infection. This sets the scene to discuss the relevance of genomic approaches, such as 16S rDNA sequencing which possess the ability to provide an extended view of the microorganisms that colonise infected DFUs. The microbiota of diabetic foot infections and role of biofilms in DFUs remains poorly understood. Understanding the microbial load, diversity and phenotypic state of microorganisms, and how they interact and or converge to bring about infection or wound chronicity in DFUs would be highly adventitious for targeted clinical therapeutics.

In chapter 3, 16S rDNA next generation sequencing was employed to profile the microbiota of infected tissues from the feet in people with diabetes. The primary aim of this study was to explore microbial communities, community structure and presence of likely pathogens from a molecular perspective, and how this extended view (if any) correlates to clinical parameters and treatment outcomes. This is imperative to determine if directed antimicrobial therapy based on conventional microbiological cultures are relevant based on genomic analysis.

The primary findings from thirty-nine patients with infected DFUs identify the duration of a DFU prior to the development of a new infective episode may be useful as a guide to directing antimicrobial therapy. The results confirm that shorter duration DFUs (<six weeks) have a

simpler microbiota consisting of one dominant bacterial species that are typically pyogenic cocci (*Staphylococcus aureus, Streptococcus agalactiae*). Conversely, longer duration DFUs (≥six weeks) are diversely polymicrobial with an average of 63 (range 19-125) bacterial species. Nineteen patients (49%) during the study period experienced antimicrobial treatment failure, but no overall differences exist in the microbiota of patients who fail therapy and those who experience treatment success (p = 0.2). Despite an extended view afforded by DNA sequencing, current guidance materials available to clinicians managing DFIs that are predominantly based on culture-dependent data are still clinically relevant and useful for treating most DFIs

In chapter 4, the primary aim was to identify the presence of biofilms in non-healing or infected DFUs. The secondary aims were to identify the accuracy of clinical cues to detect the presence of wound biofilm, as debate surrounds whether or not wound biofilms are visible to the naked eye. Accurate clinical indicators of biofilm presence in chronic wounds may aid clinicians in initiating appropriate biofilm-based treatment regimens.

Scanning electron microscopy (SEM) and peptide nucleic acid fluorescent in situ hybridisation (PNA-FISH) were employed to directly visualise microbial cells to assess the spatial organisation of microorganisms on a wound surface, and to observe any constituents of biofilm architecture such as EPS. Of 65 DFUs sampled, the presence of densely aggregated colonies (both mono and multi-species) of bacteria often surrounded by an extracellular matrix in tissue biopsies was identified in 100% of our samples. The use of binomial probabilities identified that visual cues are not better than chance alone in detecting wound biofilm, with visual cues (the presence of a gelatinous material on the wound bed, the rapid reformation of gelatinous

material after debridement, the presence of slough, and signs of pyocyanin) being absent in >50% of cases, indicating their presence is no better than chance alone.

Chapter 5 details the performance of Cadexomer iodine against microbial populations from chronic non-healing diabetic foot ulcers (DFUs) complicated by biofilm *in vivo.* Seventeen patients with chronic non-healing DFUs with suspected biofilm involvement were treated every two days for one week with Cadexomer Iodine. Tissue punch biopsies were obtained pre-and-post-treatment, with samples being subjected to molecular, microscopy and zymography methods. Microscopy confirmed the presence of dense biofilm in all patients with the application of Cadexomer Iodine being able to reduce the total microbial load by 1-2 Logs10 (p = .04), in addition to statistical reductions in wound proteases (MMP-9 – p = .03).

Analysis of the microbiota pre-and-post treatment indicate that DFUs support complex polymicrobial communities. Molecular methods demonstrate that Cadexomer Iodine has a broad level of antimicrobial activity in reducing facultative anaerobic microorganisms; *Staphylococcus* spp., *Serratia* spp., aerobic; *Pseudomonas* spp., and obligate anaerobes; *Clostridiales family XI.*

In five patients, no reduction in microbial load was noted with the use of Cadexomer Iodine. Molecular analysis was used to investigate these failed responses to therapy. Many unidentified and uncultivable microorganisms exist in DFUs. These often fluctuate from low frequency taxa pre-treatment to more dominant taxa post-treatment. The presence of “uncommon” microorganisms existing in polymicrobial biofilms may explain why some wounds are tolerant to treatment and why previously low numbers of these microbes increase when community disruption creates a nutrient availability or mutual advantage [14].

In chapter 6, an *in vitro* to *in vivo* evaluation of topical antimicrobial solutions based on a hypothesis that short exposure times are in-effective against microbial biofilms was performed. *In vitro* assessment demonstrated a variable efficacy of antimicrobial wound solutions in reducing biofilms at 15-minute exposures (0.5 log10 reductions to full eradication), with effectiveness increasing with increased exposure. Repeated exposure of hypochlorous acid in an *ex vivo* porcine skin explant model achieved < 1 log10 reduction (0.77 log10, $p = .1$), and application of 4 % w/v melaleuca oil *in vivo,* resulted in no change to the total microbial load of DFUs complicated by biofilm (median log10 microbial load pre-treatment = 4.9 log10 versus 4.8 log10 ($p = .43$).

A correlation between the poor performance of SBMO *in vitro* against *P. aeruginosa* and *S. aureus* was also seen *in vivo*. The relative abundances of *P. aeruginosa* and *S. aureus* post-treatment increased in most of the samples where it was detected. At the same time, low frequency taxa microorganisms contributing <1% relative abundance typically decreased. In these select cases, the total microbial loads pre-and-post treatment remained static. This suggests that more dominant species such as *Staphylococcus* spp. or *Pseudomonas* spp., were not affected by the topical wash, and benefitted from the increased nutrient availability[14] caused by disruption to the microbial community.

This raises an important question about the use of topical antimicrobials and their appropriate testing in *in vivo* conditions using molecular and microscopy techniques. Situations may arise where clinicians use products that result in perturbations to the complex microflora seen within chronic wounds, and this may lead to microbial dysbiosis. Of particular significance is the reduction in microbial diversity, secondary to selective actions of an antimicrobial, which may directly contribute to pathogen selection and persistence [15]. This was observed in patients in

this study where increases in known pathogens of infection (*Staphylococcus aureus* and *P. aeruginosa*) occurred following treatment.

Chapter 7, reviews the literature on current testing methods in determining the effectiveness of treatment strategies to manage biofilm. The chapter presents a discussion point that covers many of the underlying themes presented in chapters 5 and 6. Typically, biofilms show remarkable tolerance to many forms of treatments and the host immune response. This has led to vast increase in research to identify new (and to test current) anti-biofilm strategies that demonstrate effectiveness against these tolerant phenotypes. Unfortunately, a standardized methodological approach of biofilm models has not been adopted leading to a disparity between testing conditions. This has made it almost impossible to compare data across multiple laboratories, leaving large gaps in the evidence.

Furthermore, many biofilm models testing anti-biofilm strategies aimed at the medical arena have not considered the matter of relevance to an intended application. This may explain why some *in vitro* models based on methodological designs that do not consider relevance to an intended application fail when applied *in vivo* at the clinical level. This is exemplified in chapter 6, where the effectiveness of topical antimicrobial solutions tested at clinically relevant exposure times demonstrate poor effectiveness.

Chapter 8 summarises this thesis, it links chapters and presents potential future works in the area. As a collective group of works, the chapters of this thesis present a detailed exploration of managing a person with diabetes who has developed a DFU complicated by microorganisms. To identify the microorganisms that colonise and or infect tissue, 16S rDNA next generation sequencing was utilised to explore the microbiota of DFUs. Next, microscopy and molecular

techniques were utilised to better understand the phenotypic nature of microbes involved in DFUs. This identified that biofilms were ubiquitous in both infected and chronic wounds. Management of DFUs include many wound therapies such as topical antimicrobials, yet there is a lack of clinical evidence for effectiveness against biofilms. Varying topical antimicrobials were tested *in vitro, ex vivo* porcine skin explant model and in human *in vivo* clinical trials.

CHAPTER 1

1.1 FOOT INFECTION IN PEOPLE WITH DIABETES

(APPENDIX 1) **MALONE, M.** (2017) THE MICROBIOTA OF DIABETIC FOOT ULCERS AND THE ROLE OF BIOFILMS IN KON, K., & RAI, M., THE MICROBIOLOGY OF SKIN, SOFT TISSUE, BONE AND JOINT INFECTIONS (PP. 41-56). LONDON, UK, ACADEMIC PRESS, ELSEVIER.

Candidate contribution:

- ✓ Worked as the sole author for this book chapter
- ✓ Wrote the manuscript in full using information from thesis chapters 1 and 2
- ✓ Reviewed and amended all required changes and proofs

The most common pathway leading to a foot infection in a person with diabetes is through a physical break in the protective barrier of the skin, in the form of a DFU [16]. Once the skin is breached, a diabetic foot ulcer exposes the underlying soft tissues to potential bacterial colonisation with infection arising if certain conditions favour bacterial replication. Further contiguous extension to deeper structures may ensue scenarios where failure in controlling the spread of infection leads to extensive damage of host tissue and bone and this in part maybe driven by several aspects of altered immunologic function [17]. Of particular focus is the reduction in polymorphonuclear leukocyte response to bacterial infection and the role of hyperglycaemia all being reviewed [18]. This scenario optimizes the pathway to lower extremity amputation in a person with diabetes.

Bacteria that contaminate and colonize wounds likely originate from the surrounding skin flora but other sources include the environment and endogenous mucous membranes such as the gastrointestinal tract or nares [6]. One reason for this is that DFUs present an ideal environment for harbouring microorganisms since they offer a warm, moist, nutritive home, especially if devitalized tissue is present in the wound bed [6]. The longer a wound remains open the greater the chances of a more diverse and abundant bacterial colonization; with the type, depth, location, level of perfusion and the efficacy of the host immune response dictating the niche of colonizing bacteria [10,6].

Bacterial colonisation of wounds versus infection is an area that must be greatly appreciated by treating clinicians so that the appropriate use of antimicrobials and adjunct therapies can be delivered effectively. Whilst all wounds contain bacteria, colonisation refers to a specific scenario where bacteria are presently multiplying but the sum of their actions are not enough to elicit an immune response [6]. Infection and specifically DFI, has been described when

bacterial organisms proliferate within a wound (that is infra-malleolar) and in the course of doing so, cause substantial tissue damage that induces a host response accompanied by inflammation that is clinical infection [2]. For this reason the diagnosis of DFI has been promoted by expert groups as a 'clinical diagnosis' using greater than two clinical signs of infection; inflammation, erythema, local tenderness or pain, warmth and purulent discharge [2].

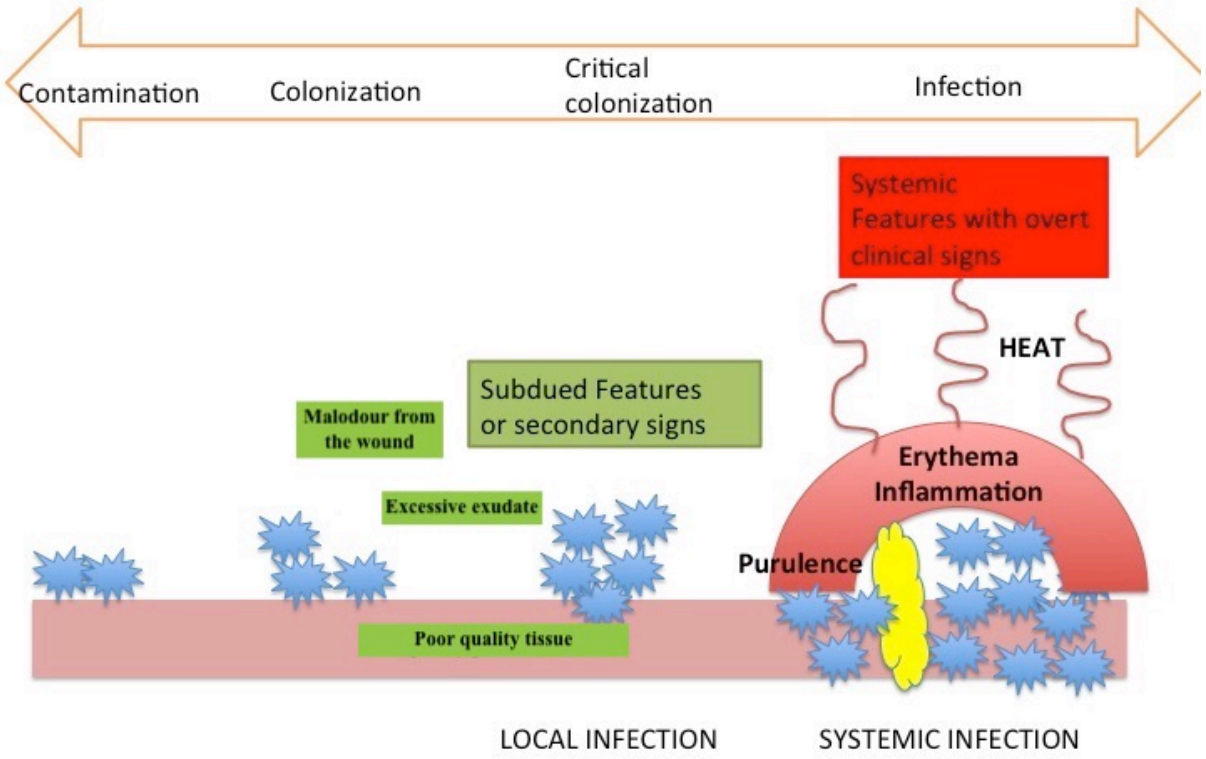
In some people with diabetes the overt clinical signs of infection are diminished or absent, and this may be due to the failure to exhibit an inflammatory response [19] with reports identifying that people with diabetes and infected chronic wounds express erythema less frequently than people without diabetes [20]. This has led to a clinical perspective that some chronic DFUs may have 'secondary signs' of infection that include but are not limited to malodour, delayed wound closure and poor quality wound bed tissue [19,21].

The fine line between colonisation and infection can be clinically challenging and some clinicians have adopted more quantitative measures to differentiate potential 'healthy' colonization from pathogenic infection by relying on the density of bacteria present per gram of tissue. Greater than 10^5 colony forming units (cfu) of bacteria per gram of tissue has been widely used as a key indicator of potential 'bioburden' as the causative factor associated with delayed wound healing. This numerical indicator is based largely on early evidence from various wound aetiologies [22] and further incorporated by others [23], yet controversy persists over whether a burden of $>10^5$ cfu of bacteria per gram of tissue is required to cause wound infection.

Kingsley (2003) [24] proposed a wound infection continuum model that placed an emphasis on the progression from colonization of bacteria within a wound through to infection. An

important component of the wound continuum model is the concept of “critical colonization” that refers to the multiplication of organisms within a wound without invasion or interfering with wound healing. Whilst the concept of critical colonization is still the center of much debate it is often used by clinicians to explain delayed wound healing in the absence of any overt clinical signs of infection and other wound delaying variables. This concept ‘if true’ may be of importance for clinicians as chronic wounds with critical colonization may benefit from local and or topical treatments such as antimicrobial wound dressings and wound debridement, rather than systemic management with the use of antibiotics, however no quantifiable data exists to prove or disprove this theory.

Figure 1.1The Infection continuum theory [22]. Adapted version for use in this thesis.



Sampling methods to detect pathogens of infected tissues

In order to guide antimicrobial therapy in the treatment of infected DFUs, clinicians must identify potential pathogens of infection using appropriate sampling techniques which are sent for conventional culture. In DFUs, wound cultures must be obtained following the removal of non-viable, devitalized tissue (either by debridement or curettage) after cleansing. The Infectious Disease Society of America (IDSA) diabetic foot guidelines and the International Working Group on the Diabetic Foot (IWGDF) diabetic foot infection committee, currently propose that tissue samples (either by curettage or biopsy) are the preferred method of collection for identifying potential bacterial pathogens from infected DFUs as they yield the most accurate results [2,25]. This is based on each expert group performing systematic reviews of the literature that identifies high-level evidence from large cohorts, that concordance between superficial swabs and tissue biopsy is low to average (range of concordance 41% - 78%) when assessed through conventional culture [2,25-28].

Conversely, some research groups have published small cohort studies identifying swabs and tissue yield similar results when exploring the microbiota of chronic wounds using either/or conventional culture and 16S rDNA sequencing [10,26,29-33]. However, there still remains scant data within the literature which have directly compared concordance between tissue and swab samples when employing solely DNA based methods from large cohorts. Debate still exists whether swab samples are equal to or superior then tissue for sampling wound microbiota.

This is exemplified in studies employing molecular techniques where biopsies from superficial and deep tissues of infected DFUs identified pathogens of infection resided in deeper tissues [34-36]. The pathogens of infection situated within deeper tissues were aerobes, along with fastidious anaerobes that were more frequently under recognized from superficial cultures.

Similarly, Frank *et al* (2014) found poor concordance (52%) between swabs and tissue specimens using16S rDNA sequencing. In only six of the 15 overlapping samples, there were high correlations between swab and biopsy samples (r = .98–1.00), while in the remaining nine of these 15 samples there were low correlations (r = .04 to .53) [37].

Advantages and disadvantages of swabs versus tissue

The major advantage for a clinician or researcher selecting a swab sampling technique is its ease of use. Whilst this technique from a clinical perspective is not promoted as the preferred sampling method for DFI, it is one of the most widely employed because of its ease of use. In this sampling method, a cotton or nylon swab is pressed firmly against the wound tissue, in the center of the wound, with the aim to express tissue fluid (containing microbes) from deep tissue layers. The technique is referred to as the Levine technique [26]. An alternate technique is where the swab traces a ten-point Z-pattern without pressure on the entire wound bed without touching the wound edge [26]. Because taking a wound swab requires little in the way of training, obtaining culture specimens through swabs is preferred by many clinicians who lack the expertise to perform a biopsy or obtain tissue specimens. Conversely, this too means that performing a swab is less invasive for patients, does not require any local anesthesia as some biopsies may require and the technique can sample a larger wound surface area [38].

Previous studies [13,29,39-41] have highlighted the potential importance of macro-spatial variation or the biogeography of how microorganisms organise themselves within wounded tissue. Sprockett *et al* (2015) reported the difference in biogeography of a wound when comparing tissue to swab, in a case report of a single patient [29]. Some of the differences identified were the bacterial load was significantly higher in samples from the wound centre than the wound edge (p = .04), yet the load at different wound depths was not significantly different.

Fazli *et al* (2009) looked at the distribution of *S. aureus and P. aeruginosa* in five chronic wounds using PNA-FISH with confocal microscopy [41]. The analysis showed that the *S. aureus* aggregates were located close to the wound surface, whereas the *P. aeruginosa* aggregates were located deeper in the wound bed ($p < .0001$).

Price *et al* (2011) performed a cross-sectional study on the macro-scale spatial variation in chronic wound microbiota in twelve patients. Curettage samples of tissue were obtained from the leading edge of the wound; the opposing leading edge; and/or the centre of the wound. While substantial macro-scale spatial variation was observed among the wounds, bacterial communities at different sites within individual wounds were similar than those in different wounds ($p = 0.001$). These results support the prevalent opinion that controlling for sample site may improve the quality of wound microbiota studies; however, the significant similarity in bacterial communities from different sites within individual wounds indicates that studies failing to control for sampling site should not be disregarded based solely on this criterion.

In summary, the evidence for obtaining tissue specimens over wound swabs when processing samples by conventional culture is clear. It is less clear when applying this to molecular techniques such as 16S rDNA sequencing. From scant reports within the literature it seems that the use of swabs maybe equal to obtaining tissue specimens when exploring the microbiota of wounds using for 16S rDNA sequencing. There are advantages and disadvantages to both methods, but ultimately larger cohort studies are required to confirm this. Macro-spatial variation is an interesting variable which could impact the overall picture of the microbiota of wounds. It seems plausible that relying on a single site for sampling such as a biopsy, may increase the chances of sampling error and that and obtaining samples from multiple sites of the wound may improve detection.

Bacteriology of Diabetic Foot Infections

The importance in managing DFIs is underpinned by the requirement to identify any pathogen/s of infection so that antimicrobial therapy can be directed. Traditional culture-dependent methods have been utilised to identify planktonic organisms subjected to controlled laboratory conditions. The limitations of such are that culture-dependent methods select for species that flourish under the controlled conditions of the diagnostic microbiology laboratory and this may not necessarily reflect the most abundant or clinically important organisms in DFIs [10].

Current culture-dependent studies on the bacteriology of DFIs have widely reported the involvement of aerobic Gram-positive cocci, mainly *Staphylococcus* spp., and *Streptococcus* spp., as being the most common aetiological agents that infect DFUs [42,43]. The spotlight has centred on the well-known pathogen *S. aureus* and its role in DFI has been well reported [16,42-45]. In two large culture-dependent studies on the bacteriology of infected DFUs [42,43] samples from over 1,266 patients indicated the predominant pathogens of infection as being *Staphylococcus aureus* and *Streptococcus* spp., with *Enterococcus* spp., and *Corynebacterium* spp., also playing prominent roles. In both studies, anaerobes were generally reported as being of low abundance with one of the studies identifying the ratio of aerobe to anaerobe isolates being approximately 7:1 [43] and the other study identifying the overall percentage of anaerobe isolates in only 28% of samples [42].

Complicating the picture of DFI is a reduced immune-mediated response (which can be present in people with diabetes), whereby the potential involvement of low virulence colonizers of devitalized tissue or bone such as coagulase-negative *Staphylococci* and *Corynebacterium* spp. may assume a more pathogenic role [46]. Additionally, whilst it is generally regarded that DFUs of short duration presenting with acute infection are monomicrobial [16], chronic infected DFUs

seem to harbour a much more complex polymicrobial flora including *Pseudomonas* spp., Enterococcus spp., Enterobacteriaceae family, and other obligate anaerobes [42,43,47,48].

1.2. HUMAN SKIN

1.2.1. THE SKIN IS OUR FIRST LINE OF DEFENCE AGAINST INVADING MICROORGANISMS.

Human skin is involved in an array of processes that include but are not limited to forming an outer protective shell that acts as a barrier to external threats. In addition to that of a barrier, the skin plays an integral role as an immunological interface that modulates the residing microorganisms that constitute the skin flora [49] who for the most part reside as non-pathogenic permanent residents (commensal flora). The fine balance between the host and microbe is essential for the propagation of mutual benefits (symbiosis and or commensalism) and this may afford protection against invasion from more pathogenic bacteria. One such example of this beneficial relationship is the proposed presence of the commensal bacterium *S. epidermidis* and its ability to inhibit the known pathogen *S. aureus* [50].

The epidermis forms the outermost layer of the skin and keratinocytes (KCs) constitute the predominant cells providing an essential first line of innate immunity [51-52]. Specific receptors present within KCs known as pattern recognition receptors (PRRs) are stimulated by pathogenic organisms intent on invading the epidermis via surface associated molecular structures [53]. Examples of PRRs are the Toll-like receptors (TLRs) that detect pathogen-associated molecular patterns (PAMPs) such as the lipopolysaccharide (LPS) of Gram-negative bacteria or the peptidoglycan (PGN) of Gram-positive bacteria [54]. Our current understanding

of the mechanisms of action of TLRs is the vital role they play in the induction of antimicrobial responses, with a myriad of TLRs being reported in the literature, each with their own unique function [54]. A study by Jugeau *et al* (2005) demonstrated that *P. acnes* induced expression of TLR-2 and TLR-4 by KCs in addition to MMP-9, a known pro-inflammatory protease [55]. TLR-2 has also been identified as crucial for the pro-inflammatory signalling pathway activating NF-kB through the myeloid differentiation protein MyD88 [54].

The epidermis perpetuates other defence mechanisms in response to invading pathogens such as the process of KC up-regulation and expression of endogenous antimicrobial peptides (AMPs) in response to inflammatory stimuli. Over 20 AMPs have been reported on human skin [56] where they pose a formidable broad-spectrum antimicrobial activity, however typically they can be sub-divided into two families; β-defensins (hBD) [57] and cathelicidins [58].

Cathelicidin has been purported to be an important regulator of new born babies' microbiota through its over-abundance in newborns where it has been shown to significantly inhibit the growth of *S. epidermidis* [59], whilst hBD exhibits a broad range of antimicrobial activity against Group A Streptococci, *S. aureus*, *E. coli* and *P. aeruginosa* [60]. In a study by Percoco *et al* (2013) [61] utilising real time qPCR, human epidermal cells exposed to the bacterial stimuli of *S. epidermidis* and *P. fluorescens* resulted in the increased transcription of the genes that encode for AMPs (hBD) in tandem with increased expression of pro-inflammatory cytokines interleukin (IL)-1α and (IL)-1-β, as well as IL-6.

Specifically, the role of KCs as regulatory orchestrators of cutaneous inflammation and immunity through cytokine and chemokine activity is noteworthy. Even more so is the inherent ability to mobilise a defensive strike against invading pathogens while abstaining in the

presence of ‘friendly fire’. In some instances, this friendly fire or commensal enterprise can be of benefit. One recent study has identified the mutual benefit of *S. epidermidis* and the phenol-soluble modulins that it produces, which can selectively inhibit the more pathogenic *S. aureus* and even co-operate with host AMPs to improve bactericidal activity [62]. Expanding this concept has been the recent body of work suggesting commensal microorganisms may have a role in modulating the innate immune response through ‘cross-talking’. Lai *et al* (2010) reported on the inhibition of TLR-2 and TLR-3 through a mediated cross-talk mechanism driven by lipoteichoic acid production by *S. epidermidis* [60]. The same group also identified the ability of *S. epidermidis* to incite KCs expression of AMPs through the TLR-2 pathway.

Taken collectively, the evidence above presents the extensive role that KCs, the epidermis and microorganisms play in the innate immune response. Several teams have observed the links between KCs and the innate response to microbial recognition and have postulated cross talking between the innate pathways, microorganisms and KCs. Considering this, the role of the epidermis as an adjuvant is distinguishable and of importance. The loss of this function would clearly pose a risk of infection to a person with diabetes.

1.2.2. THE HUMAN MICROBIOME PROJECT

Within the last decade, groups of researchers have actively investigated the role and impact of microorganisms on human health and disease through the human microbiome project. This project involves the identification of all known microbes residing on and in the human host, including; bacterial, viral, fungal and archaea. The human microbiome project is a consortium of nearly 80 universities and scientific institutions who have mapped the microbial makeup of

the human body using molecular approaches and in doing so, created reference databases that have provided a foundation to accelerate infectious disease research [63].

Gao *et al* (2007) [64] employed PCR-based DNA sequencing to survey the skin microbiota of the volar forearm in 6 healthy subjects. Actinobacteria, Firmicutes and Proteobacteria contributed to 95% of sequences. Similarly Grice and colleagues (2008) [7] examined the diversity of the microbiota of human skin identifying Proteobacteria (59%) as the dominant bacterial Phylum. Utilising amplification and sequence analysis of 16S rDNA, Grice and colleagues (2008) [7] obtained skin samples (swab, scrape and tissue biopsy) from five healthy volunteer's arms and grouped the DNA sequences into operational taxonomic units (OTUs). In the context of molecular methods OTUs are clusters of single DNA sequences grouped together based on their similarity to other sequences in the community and used to define microorganisms to species or genera level. The most abundant OTUs belonged to the genus *Pseudomonas* spp., a member of the Proteobacteria Phylum followed by *Janthinobacterium* spp. Other OTUs identified belonged to the phyla of Actinobacterium (28%), Firmicutes (12%) and Bacteroidetes Phyla (9.7%).

With the advent of the human microbiome project and the evolution of culture independent methods employing molecular techniques, researchers have now identified the majority of all microorganisms residing on healthy adults. In contrast, environmental microbiologists estimate less than 2% of all bacteria are known via cultivation based approaches [65].

Human skin is more than an external barrier, it is composed of unique microbial communities that vary depending on anatomical location. The skin microbiota may house core microorganisms that constitute a steady state of symbiosis [66]. If the skin barrier is disrupted,

such as occurs in DFUs, symbiosis may shift to parasitic invasion, especially if the host may already be immunocompromised. It is therefore quintessential to understand what microorganisms reside on intact healthy skin and to understand their potential for causing human disease.

1.2.3 IS NATIVE SKIN FLORA DIFFERENT IN PEOPLE WITH DIABETES VERSUS NON-DIABETIC

Bacteria that contaminate and colonize wounds likely originate from the surrounding skin flora but other sources include the environment and endogenous mucous membranes such as the gastrointestinal tract or nares [6]. There is evidence that the native skin flora in the feet of people with diabetes are different than those of non-diabetics. Using 16s rDNA sequencing, Redel *et al* (2013) compared intact skin in the feet of people with and without diabetes (healthy control). In the feet of people with diabetes, intact skin demonstrated increased populations of *S. aureus* with reduced quantities of other staphylococci genera. Bacterial diversity when compared to healthy controls was also greater in the feet of people with diabetes [67].

Oates *et al* (2013) employed PCR-denaturing gradient gel electrophoresis (DGGE) in conjunction with DNA sequencing to compare intact (healthy) skin in the feet of 26 people with diabetes. In skin samples, the most prevalent genera were Staphylococcus spp. followed by the class Bacilli [68]. Similarly, Gardiner *et al* (2017) [32] used 16S rDNA sequencing to also compare the microbiota of intact (healthy) skin in the feet of persons with diabetes versus non-diabetics (controls). In the feet of people with diabetes the skin was significantly less diverse than non-diabetics and the community composition was also significantly different between the two. Despite the differences in community composition the most abundant taxa from both groups were similar. Both groups were dominated by the genera *Staphylococcus* spp.,

Acinetobacter spp., and *Corynebacterium* spp [32]. The above data may help to support a notion that diabetes-specific alterations in normal/native flora predispose diabetic patients to greater exposures of colonization by specific species, such as the highly virulent *S. aureus*. This also supports existing data that indicate many of the aforementioned microorganisms as common pathogens of DFI.

1.3 16S rDNA next generation sequencing

(Appendix 2) Malone, M., Gosbell, I. B., Dickson, H. G., Vickery, K., Espedido, B. A., and Jensen S. O. Can molecular DNA-based techniques unravel the truth about diabetic foot infections? Diabetes Metabolism Research and Reviews 2017. 33: e2834.DOI: 10.1002/dmrr.2834.

Candidate contribution:

- ✓ Worked as primary author for publication
- ✓ Wrote the manuscript in full using information from thesis chapters 1 and 2
- ✓ Designed and produced Table 1 and Figure 1
- ✓ Reviewed and amended all required changes from co-author contributions
- ✓ Submitted the manuscript as primary author

1.3.1. INTRODUCTION

Diabetic foot infections (DFI) are a common complication when breaks in the protective barrier of the skin occur in people with diabetes allowing easy entry of bacteria. A diabetic foot ulceration (DFU) that becomes infected is a major causal pathway to lower extremity amputation and the identification of causative pathogens and any accomplices is vital in directing antimicrobial therapy. Historically, clinicians have relied upon culture-dependent techniques that are now widely acknowledged as both being both selective for microorganisms that thrive under the physiological and nutritional constraints of the microbiology laboratory, and that grossly underestimates the microbial diversity of a sample.

The amplification and sequence analysis of 16S rDNA gene has revealed a vast diversity of microorganisms in DFIs extending the view of the diabetic foot microbiota. The interpretation of these additional findings and their relevance to clinical care remains largely unexplored. The advancement of molecular methods that are culture-independent and employ DNA sequencing technology represent a potential "game changer". One advancement with the potential to provide a greater understanding of infection is the role of metagenomics and its shotgun approach to surveying whole-community genomic DNA from within a sample (whole genome shotgun sequencing, WGS) [69,70].

In this respect "whole genome shotgun sequencing" affords the possibility to characterize not only the microbial diversity within a DFI (*i.e.,* "which microorganisms

are present") but the biological functions of the community such as virulence and pathogenicity (*i.e.*, "what are the microorganisms capable of doing") [71]. This review will focus on interpreting the current scientific evidence that is available about new molecular techniques for exploration of the microbiota of infected and uninfected DFUs, exploring the potential of these new technologies and postulating how they could translate to improved clinical care.

1.3.2. CULTURE-DEPENDENT DFU BACTERIOLOGY

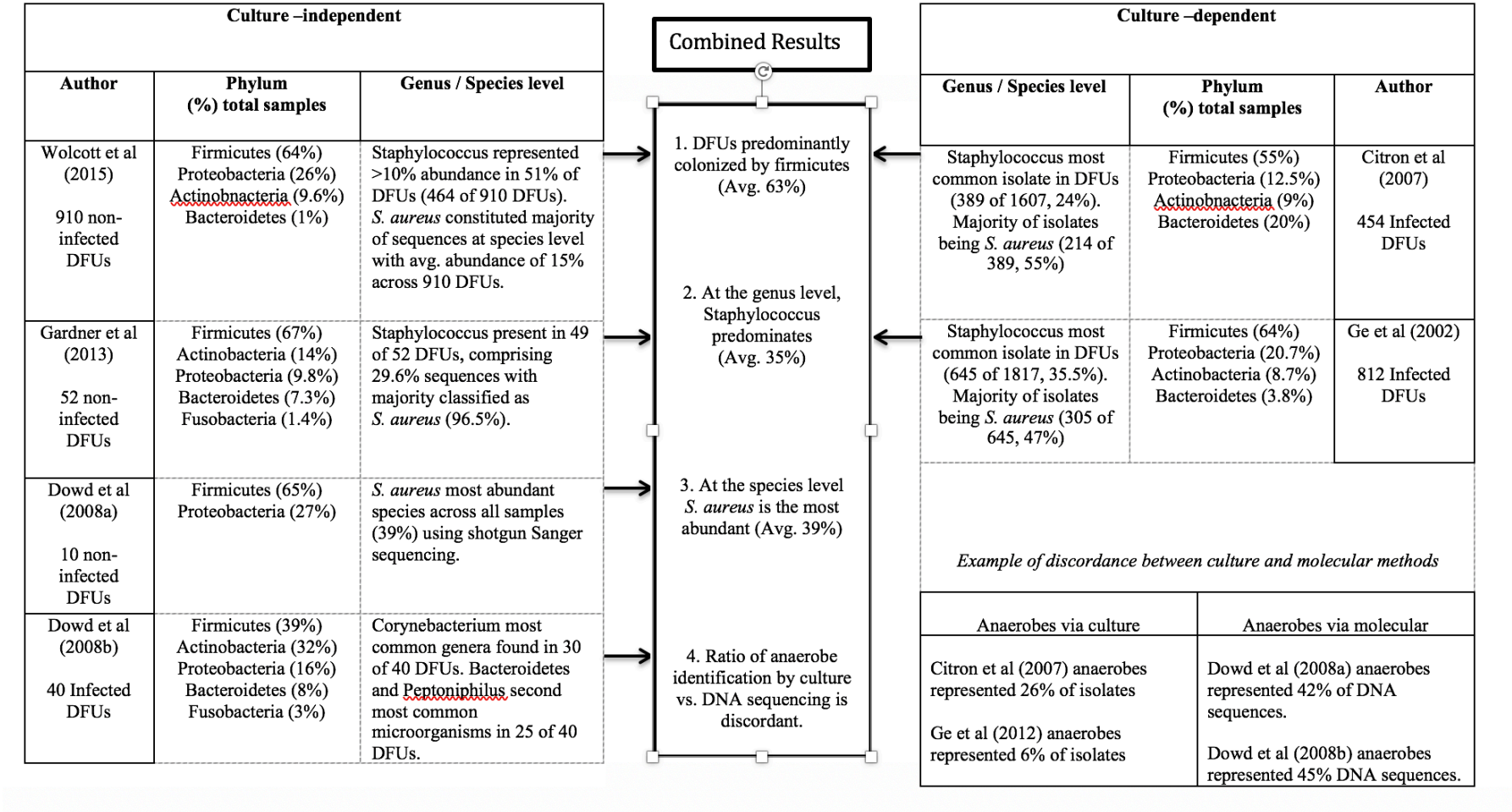
The importance in managing DFIs is underpinned by the requirement to identify any pathogen so that antimicrobial therapy can be directed. Traditional culture-dependent methods have been utilised to identify planktonic organisms subjected to controlled laboratory conditions. Many bacteria that can be grown in the laboratory are only a small fraction of the total diversity that exists in nature. Importantly, bacterial species can exist in a viable but non-culturable state [72]. The limitations of this are that culture-dependent methods select for species that flourish under the conventional culture methods utilised in the routine diagnostic microbiology laboratory, and this may not necessarily reflect the most abundant or clinically important organisms in DFIs [7]. This could lead to an underestimation of total viable cells within a clinical sample posing an increased risk to the patient.

The majority of studies exploring the bacteriology of DFIs have utilised conventional culture methods. These have widely reported the involvement of aerobic Gram-positive cocci, with *S. aureus* and *Streptococcus* spp., being the most common aetiological

microorganisms that acutely infect DFUs (Table 1.1) [42,43]. The spotlight has centred on the well-known pathogen *S. aureus* and its role in DFI has been well reported [25,42].

In two large culture-dependent studies on the bacteriology of infected DFUs samples from over 1,266 patients indicated the predominant pathogens being *S. aureus* and *Streptococcus* spp., with *Enterococci* spp., and *Corynebacterium* spp., also playing prominent roles [42,43]. In both studies, anaerobes were generally reported as being of low abundance with one of the studies identifying the ratio of aerobe to anaerobe isolates being approximately 7:1 [43] and the other study identifying anaerobe isolates in only 28% of samples [42]. In DFI, patients may also exhibit reduced immune-mediated responses, where the potential involvement of low virulence colonizers of devitalized tissue or bone, such as coagulase-negative staphylococci and *Corynebacterium* spp., may assume a more pathogenic role [46]. Additionally, while DFUs of short duration presenting with acute infection are generally monomicrobial [16], chronic infected DFUs seem to harbour a much more complex polymicrobial flora including *Pseudomonas* spp., *Enterococcus* spp., *Enterobacteriaceae* family., and other obligate anaerobes [42,46,47].

Table 1.1 Bacteriology of culture-dependent and independent studies of infected DFU



Culture –independent

Author	Phylum (%) total samples	Genus / Species level
Wolcott et al (2015) 910 non-infected DFUs	Firmicutes (64%) Proteobacteria (26%) Actinobnacteria (9.6%) Bacteroidetes (1%)	Staphylococcus represented >10% abundance in 51% of DFUs (464 of 910 DFUs). *S. aureus* constituted majority of sequences at species level with avg. abundance of 15% across 910 DFUs.
Gardner et al (2013) 52 non-infected DFUs	Firmicutes (67%) Actinobacteria (14%) Proteobacteria (9.8%) Bacteroidetes (7.3%) Fusobacteria (1.4%)	Staphylococcus present in 49 of 52 DFUs, comprising 29.6% sequences with majority classified as *S. aureus* (96.5%).
Dowd et al (2008a) 10 non-infected DFUs	Firmicutes (65%) Proteobacteria (27%)	*S. aureus* most abundant species across all samples (39%) using shotgun Sanger sequencing.
Dowd et al (2008b) 40 Infected DFUs	Firmicutes (39%) Actinobacteria (32%) Proteobacteria (16%) Bacteroidetes (8%) Fusobacteria (3%)	Corynebacterium most common genera found in 30 of 40 DFUs. Bacteroidetes and Peptoniphilus second most common microorganisms in 25 of 40 DFUs.

Combined Results

1. DFUs predominantly colonized by firmicutes (Avg. 63%)
2. At the genus level, Staphylococcus predominates (Avg. 35%)
3. At the species level *S. aureus* is the most abundant (Avg. 39%)
4. Ratio of anaerobe identification by culture vs. DNA sequencing is discordant.

Culture –dependent

Genus / Species level	Phylum (%) total samples	Author
Staphylococcus most common isolate in DFUs (389 of 1607, 24%). Majority of isolates being *S. aureus* (214 of 389, 55%)	Firmicutes (55%) Proteobacteria (12.5%) Actinobnacteria (9%) Bacteroidetes (20%)	Citron et al (2007) 454 Infected DFUs
Staphylococcus most common isolate in DFUs (645 of 1817, 35.5%). Majority of isolates being *S. aureus* (305 of 645, 47%)	Firmicutes (64%) Proteobacteria (20.7%) Actinobacteria (8.7%) Bacteroidetes (3.8%)	Ge et al (2002) 812 Infected DFUs

Example of discordance between culture and molecular methods

Anaerobes via culture	Anaerobes via molecular
Citron et al (2007) anaerobes represented 26% of isolates Ge et al (2012) anaerobes represented 6% of isolates	Dowd et al (2008a) anaerobes represented 42% of DNA sequences. Dowd et al (2008b) anaerobes represented 45% DNA sequences.

1.3.3. CURRENT STUDIES EMPLOYING MOLECULAR TECHNIQUES FOR DIABETIC FOOT INFECTIONS AND CHRONIC WOUNDS

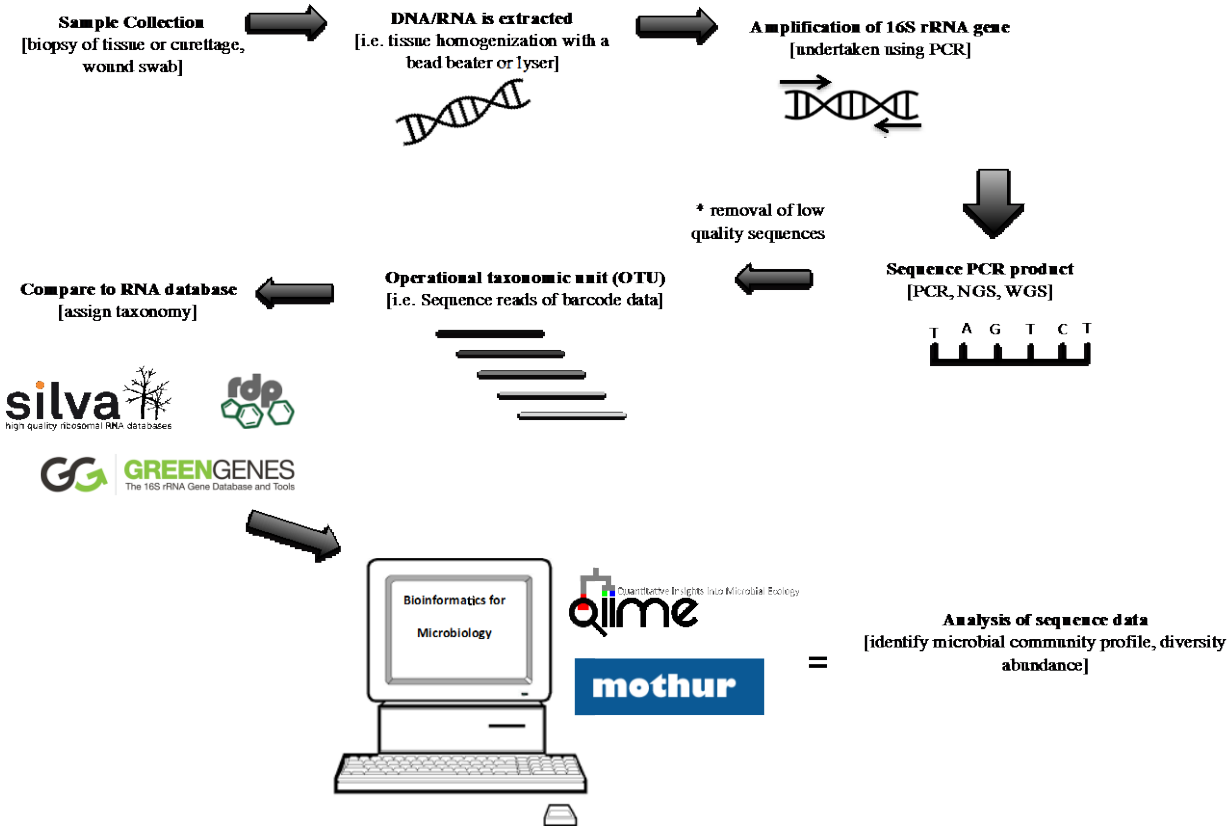
The advent of molecular DNA based techniques that are culture-independent have identified limitations of traditional culture-dependent methods, acknowledging their capacity to identify a limited number of known cultivable bacteria (less than 1%) [7]. DNA-based techniques, supported by ever-growing gene reference libraries, allow for the characterization of microbial communities or microbiota's that encompass the sum of all microorganisms residing on and within the host - bacterial, fungal and viral [73].

Characterizing the microbiota of infected and uninfected DFUs using molecular methods is growing with increasing publications occurring year on year [8-10,74]. Composite evidence by authors employing molecular techniques also exist for chronic wounds, where studies have pooled multiple wound aetiologies to provide an overview of the chronic wound microbiota [75-78]. Most chronic wound microbiota studies to date have employed pyrosequencing centred approaches that amplify and sequence the small subunit ribosomal RNA (16S rRNA) gene, a highly-conserved gene present in all prokaryotic DNA (bacteria) but not eukaryotes (humans). This has revealed a vastly more complex array of bacterial communities than those identified by traditional culture-dependent methods.

16S rDNA is an ideal target for bacterial DNA analysis given that it possesses nine hypervariable regions that have considerable sequence diversity between bacterial taxa [79]. The hypervariable regions are bordered by stretches of sequences that are highly conserved between bacteria, ideal for designing universal and species-specific primers

to amplify the hypervariable regions by PCR [80]. The workflows required to generate data on microbiota studies that include chronic wounds are depicted in Figure 1.2

Figure 1.2 An overview of a common PCR and MPS-based workflow employed for microbiota research into chronic wounds



The first report in the literature on the microbiota of DFUs was undertaken by Dowd *et al* (2008a) reporting on ten chronic DFUs using multiple genomic approaches that included; partial ribosomal amplification and pyrosequencing (PRAPS), full ribosomal amplification, cloning and Sanger sequencing (FRACS), density gel electrophoresis (DGGE) and Sanger sequencing (PRADS) [8]. Identification of species was undertaken using operational taxonomic units (OTUs), these are clusters of single DNA sequences

grouped together based on their similarity and used to define microorganisms to a species or genus level.

DNA reads from each wound were compiled to create a pooled DNA data set from multiple DFUs. Facultative and strict anaerobic Gram-positive cocci formed most sequences with genus level identification highlighting the predominance of *Staphylococcus* spp., (PRAPS = 10874 of 36508 sequences, 30%, FRACS = 70 of 178 sequences, 39%) in addition to *Peptoniphilus* spp., *Anaerococcus* spp., *Rhodopseudomonas* spp., *Enterococcus* spp., *Veillonella* spp., *Bacterioides* spp., and *Finegoldia* spp., each contributing to greater than 5% of the microbial diversity.

The second study to explore DFU microbiota was undertaken by Dowd *et al* (2008b) who employed bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) to sample 40 chronic infected DFUs from a range of locations on the foot and ankle [9]. Dowd *et al* (2008b) hypothesized that a single major pathogen would be associated with all wounds therefore DNA reads for each DFU were reported individually and not pooled. This allowed the compilation of community profiles for each DFU including the accurate identification of the number of samples each genus was detected, and the average percentage each genus contributed to its positive sample.

Results identified each DFU contained a rich diversity of microorganisms with *Corynebacterium* spp., (30 of 40 DFUs, avg. abundance = 14.4%), *Bacteroides* spp., (25 of 40 DFUs, avg. abundance = 24.2%), *Peptoniphilus* spp., (25 of 40 DFUs, avg abundance = 13.6%), *Fingoldia* spp., (23 of 40 DFUs, avg. abundance = 6.7%) *Anaerococcus* spp., (22 of 40 DFUs, avg. abundance = 7.7%) and *Streptococcus* spp.,

(21 of 40, avg. abundance = 36.5%) being present in all samples. Surprisingly, *Staphylococcus* spp., was only present in 13 of 40 (avg. abundance = 8.3%) DFUs compared to a previous study by the same authors that identified *Staphylococcus* spp., as the major bacterial genus in 10 infected DFUs [8].

The location of ulcers originating from multiple sites of the foot and ankle may have increased the heterogeneity observed between samples in the latter study. Some DFUs may have been purely ischemic or neuroischemic in origin, increasing the likelihood for the presence of devitalized, hypoxic tissue and selection towards low-virulent colonizers and anaerobic microorganisms.

No single genus of bacteria was present in all 40 DFUs and this led Dowd *et al* (2008b) to hypothesize the microbiota of DFUs (and chronic wounds) is dominated by multiple species which cooperate in a biofilm; a concept Dowd *et al* 'coin' as functionally equivalent pathogroups. This centers on genotypically distinct bacteria working cooperatively to induce pathogenesis similar to that observed from a known single pathogen, such as *S. aureus*; however, there are currently limited data available to support this.

Gardner *et al* (2013) [10] profiled the microbiotata of 52 individuals with non-infected DFUs using 16S rDNA sequence analysis and were the first to restrict the sampling of patients to a homogenous sample of DFUs (neuropathic DFUs only). The group proposed that previous studies within the area were restricted by heterogeneous sampling (*e.g.*, pooled samples of differing chronic wounds and of all three DFU aetiology types (neuropathic, ischemic and neuroischemic) and, as such, these

pathophysiologically distinct lesions likely harbour their own unique microenvironments and thus microbiota [8,9].

In characterizing the microbiota of 52 non-infected DFUs, a total of 13 Phylum were reported from 300,660 DNA sequences with the majority being classified into Firmicutes (67%), Actinobacteria (14%), Proteobacteria (9.8%), Bacteroidetes (7.3%) and Fusobacteria (1.4%). *Staphylococcus* spp., was identified as the most common and abundant genus in 49 of 52 DFU samples (DNA sequences = 88,995 of 300,660, abundance = 29.6%). At a species-level the majority of sequences belonged to the common pathogen *S. aureus* (*S. aureus* sequences = 85,880 of 88,995, 96.5%), an unsurprising finding considering the highly documented role of this microorganism in diabetes related foot infection [2].

Further analysis of microbial diversity in 52 DFUs reported on average 30 OTUs per DFU (range 7 – 64) in comparison to culture-dependent analysis that detected on average 4 isolates per DFU ($p < .0001$). Comparisons of the relative bacterial abundances within each DFU using culture-dependent analysis identified the overestimation in the abundance of *Staphylococcus* spp., (.47 vs. .32, $p < .0001$) and underestimation of anaerobes (.11 vs. .18, $p = .0063$) in comparison to 16S rDNA sequence analysis.

By culture, anaerobes were identified as the predominant organisms in only 6 of 52 DFUs (12%) a finding consistent with the known limitations of culture-dependent methods, particularly in the identification of slow-growing, fastidious anaerobic organisms [10]. In contrast, 16S rDNA sequence analysis identified twice this amount (12

DFUs, 23%); this is of importance at a clinical level where the role of anaerobes in DFI and wounds is often underestimated. Their significance as highly virulent pathogens, detrimental to the wound environment [6], or as pathogens of DFI [47] is only becoming appreciated through advances in new molecular techniques that are culture-independent.

Gardner *et al* (2013) also depicted bacterial community structure using a statistical approach of Euclidean distances to determine how similar DFU attributes were to the indicators of wound bioburden (microbial load, diversity and presence of pathogens). The partitioning of data identified three clusters in which Gardener *et al* (2013) referred to as EUC1, EUC2, and EUC3. EUC1 contained significantly greater OTU richness (p = .006), diversity (p = .02) and bacterial load (p = .02), whereas EUC2 contained a greater abundance of *Staphylococcus* spp., and lower abundance of anaerobes (p = .0003) and EUC3 contained the highest abundance of *Streptococcus* spp., (p = .0002).

Euclidean clusters were also mapped against clinical markers that included HbA1c, mean tissue oxygenation, ulcer duration, ulcer depth, ulcer surface area and necrotic tissue. EUC1 containing greater OTU richness, diversity and bacterial load, were associated with DFUs of longer duration (p = .02) and increased ulcer depth (p = .01). EUC1 also contained a greater abundance of anaerobes (p = .01) and Proteobacteria (p = .005), a likely association with wound longevity and deeper tissue involvement. EUC 2 and EUC 3 had the highest proportions of both *Staphylococcus* spp., and Streptoco*ccus* spp., with laboratory markers from these participants recording the highest levels of glycosylated haemoglobin (HbA1c %) (Median EUC 2 HbA1c = 9.2% versus median EUC 3 HbA1c = 9.4%).

Assumptions on infections in people with diabetes have repeatedly associated *Staphylococcus* spp., and *Streptococcus* spp., as major pathogens, noting higher colonisation rates in those with sub-optimal glycaemic control [6,81,82]. A plethora of studies have also identified that hyperglycaemia causes immunosuppression, and a reduction in glucose by a variety of means reverses immune function deficits [83-85]. However, no evidence exists identifying direct links between hyperglycaemia, infection and potential deficits in the immune response to specific microorganisms [2,86].

Gardner *et al* (2013) invested a significant proportion of their analysis on undertaking a statistical approach to partitioning DFUs into meaningful clusters based on their associations to the three dimensions of bioburden (*i.e.,* microbial load, microbial diversity, presence of pathogens). Interpretation and validation of cluster analysis occurred through a silhouette score (SI), a method used to report the graphical representation of how similar one cluster is in comparison to other clusters. An average SI score of .42 was reported by Gardner *et al* (2013), but a SI score of less than < .5 signifies only modest support for the proposed cluster algorithms [87,88]. Other researchers such as Wu *et al* (2011) [89], who explored gut microbial enterotypes using molecular approaches, recommended using a high SI threshold (≥ .75) for validating clusters.

The statistical significance of partitioning 52 DFUs into similar clusters given the moderate SI score should be aired on the side of caution as a low SI score suggests that clustering could be due to chance. Further studies aiming to increase cluster validity

through higher SI scores may be required in supporting the partitioning of DFU microbiota to clinical indicators like those observed by Gardner *et al* (2013).

Wolcott *et al* (2015) retrospectively reported 910 non-infected DFUs using 16S rDNA sequencing and currently represents the largest body of evidence surveying the microbiota of DFUs [90]. Wound samples were collected from superficial debridement material obtained from the wound bed and relative abundances were calculated from species-level OTUs constituting >10% of all DNA reads per sample. Given the large sample size from multiple wound aetiologies, individual wound microbiotas were not reported, and DNA sequences were pooled. Of 910 DFUs the most abundant Phyla were Firmicutes (64%) followed by Proteobacteria (26%), Actinobacteria (9.6%) and Bacteroidetes (1%). *Staphylococcus* spp., (31%) was the most abundant OTU with *S. aureus* (48%) and *S. epidermidis* (35%) predominating at the species-level.

Smith *et al* (2016) [74] sampled 20 new and recurrent DFUs using superficial swab cultures and analysed the data through 16S rDNA sequencing. They hypothesized that distinct differences would exist between the microbiota of new ulcer versus those that were recurrent. Herein lies one of the major methodological flaws of this study, of which there are several. Smith *et al* (2016) fail to define what a recurrent ulcer is. Logically one would assume recurrent ulcers are healed wounds that due to factors such as peripheral neuropathy and altered foot architecture, eventually at a time point re-ulcerate. But these are then classified as new ulcers as the skin barrier was intact prior to re-ulceration. Therefore, there is no real difference between the two cohorts examined.

This point is proven as the study identified that new ulcers were predominated by *Peptoniphilus* spp., (6 samples), *Staphylococcus* spp., (5 samples), *Anaerococcus spp*., (5 samples) and *Corynebacterium* spp., (4 samples). Recurrent ulcers had similar microorganisms; *Corynebacterium spp*., (5 samples), *Peptoniphilus* spp., (4 samples) and *Anaerococcus* spp., (4 samples). Further analysis of both alpha and beta diversity (dominance, diversity, principal coordinates analysis) identified no differences in the microbiota of either wound.

Another drawback of the study is the poorly presented data which makes comparison to other studies difficult. The primary table of results that identify genera through 16S rDNA sequencing are not presented as rank abundance, instead the authors name genera alphabetically and omit reference to the relative abundance of microorganisms identified. This severely restricts the ability to decipher community structure and determine who the major or minor players are in these wounds and if any differences existed between groups.

The omission of data pertaining to the infection status or chronicity of wounds is further limiting. Given that most DFUs will be colonized by microorganisms on injury, this does not necessarily translate to microbial pathogenesis. If DFUs are appropriately managed through standard care (i.e. offloading and woundcare) and if the host response controls colonization, these DFUs are likely to heal [91]. Had the authors compared the microbiota of new wounds that healed in the study groups to wounds that failed to heal, then this would have been novel to the literature and may have increased our understanding of the wound microbiota.

The major defining features linking all the above studies are that samples were obtained at singular time points. This does not allow for the temporal analysis of wounds sampled longitudinally which may identify shifts in community structure or diversity that may lead to impaired healing or clinical infection. Importantly monitoring temporal shifts may allow for model prediction of wounds which heal, remain chronic or become infected. Recently three studies have employed these methods to explore DFUs [92,30,31].

Loesche *et al* (2017) [31] examined 100 participants with neuropathic DFUs, obtaining bi-weekly superficial swab cultures over 26 week follow up period. *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp., and *Anaerococcus* spp., were the most abundant genera in DFUs. Previously this group had used using partitioning around medoids [10], to identify specific clusters of microbiota from DFUs with similar traits. In the new temporal study, the authors utilised Dirichlet multinominal mixture (DMM) model-based approach to cluster longitudinal DFU samples in 4 groups or community types (CT). CT1 and CT2 were highly heterogeneous with no dominant taxa, CT3 were characterized as having high relative abundance of *Streptococcus* spp., and CT4 DFUs had high relative abundances of *S. aureus*.

The community type clusters allowed further analysis against longitudinal samples, where transitions or shifts of the microbiota were apparent. Wounds that healed faster (<12 weeks) experienced greater transition frequencies than wounds which took longer to heal (>12 weeks) ($p < .0001$), of which these wounds were dominated by CT1 and CT2. CT3 and CT4 experienced stability with minimal transitions and these wounds took longer to heal. The authors proposed therefore that their findings suggest community stability reflects a delayed healing phenotype.

From the same 100 patient sample, Kalan *et al* (2017) [31] employed high through-put sequencing to profile the fungal mycobiome using the internal transcribed spacer (ITS1) region. They identified that up to 80% of the 100 DFUs contained fungi, with the entire dataset represented by two phyla; *Ascomycota* and *Basidiomycota*. The most abundant species were *Cladosporidium herbarum, Candida albicans* and *Trichosporon* spp. The authors additionally correlated clinical data against fungal mycobiome and identified increased relative abundance of fungi after antibiotic therapy and increased fungal diversity in DFUs with poor perfusion. Kalan's study further tried to correlate the fungal mycobiome with clinical outcomes but identified no significant correlations. Explanations as to lack of clinical correlation with outcomes were provided in an open letter by Malone & Dickson (2017) (Appendix 3)

Kalan *et al* (2016) report only on the ITS1 sequences (18S rDNA) and do not include bacterial or archaeal sequences (16S rDNA) paying reference to the composite relative abundances of each group. In doing so, the clinical relevance of fungi in chronic wounds becomes lost. This is because, without identifying all the microorganisms within a wound (bacterial, fungal, archaeal), one cannot determine the overall microbial load for fungi or what their relative abundances are in relation to those of other microorganisms. This allows us to understand whether a microorganism is a dominant, major, or minor player. Therefore, no assumptions can be made on the community structure, and the "mycobiome" becomes clinically uninterpretable.

The data presented by Kalan *et al* (2016) are difficult to interpret within the context of clinical management. They report the sampling of chronic wounds undertaken using the Levine technique with a swab. This culture method has been the subject of great

debate in the diabetic foot arena, with opinions still divided. Some expert groups promote tissue biopsy as the most appropriate sampling method for identifying pathogens of infection and for exploring both the microbiota and the role of biofilms [93,25].

A previous study from the authors' group suggests good concordance between culture-independent swab samples (DNA sequencing) and tissue samples [26]. However, the use of swab samples from superficial tissue makes it difficult to ascertain whether any fungi identified merely resided on wound surfaces as colonizers or whether the fungi were invasive and involved deeper tissue (this may suggest a more pathogenic involvement). Tipton *et al* (2017) [73] obtained DFU samples from three-time points in 167 subjects from a previously reported sample dataset [90,92]. Community stability was quantified as the percentage of genera/species observed at each time point that were still present at subsequent time points. Dirichlet multinominal mixture (DMM) model-based approach was used in a similar fashion to Loesche *et al* (2017) [31], to cluster wound communities into community types. The most prevalent species identified as common and major contributors (>1% to >10%) in DFUs were *S. aureus*, *P. aeruginosa*, *S. haemolyticus*, *C. straiatum*, *S. epidermidis* and *F. magna* which accounted for 41% of total microorganisms.

When combining samples longitudinally, a trend was observed between time points where low frequency taxa (<1%) later contributed to DFU microbiota at higher relative abundance levels (>10%) in 20% of DFUs. Community types determined through DMM did not identify any clear pattern of clusters with the exception of clusters that were highly variable akin to those identified by Loesche *et al* (2017) [31] of CT1 and

CT2. This variability is likely due to the data by Tipton *et al* being affected by treatment variables that patients received, such as the use of antibiotics, or by the time between sample intervals which was around 3 months (significantly longer than Loesche *et al* (2017) who performed sampling bi-weekly.

The prospect of understanding changing shifts in the microbiota of wounds that may enable predictive modelling is compelling and would likely significantly impact patient care and therapeutics. There are some potential limitations that may restrict its ability to be used on a generalised scale. Firstly, analyzing single samples from >100 people that are pooled together, by itself introduces large heterogeneity into the dataset. This may skew the data and cloud highly relevant pictures that would otherwise be visible at the individual wound level. When combing multiple samples from patients, this may further complicate the heterogeneity of samples. Other variables which may skew data and introduce error are sampling methods and macro-spatial variation [13]. Loesche *et al* (2017) [31] utilised superficial swabs which can capture the full area of a wound surface when using the Z technique or the central wound area when using the Levine technique. However, expert groups suggest that superficial swabs are not appropriate for depicting the full array of microorganisms residing in a wound [2,25].

Tissue samples are promoted as the preferred detection methods, yet many tissue punch biopsies or debridement material may only capture a specific area of the wound and thus miss areas of microbes that contribute to the microbiota [41]. These factors may add to the sampling error of data and restrict the generalisation of modelling or the interpretation of data from large cohorts. Lastly, what the two temporal analysis studies fail to achieve is to capture patients where the microbiota of wounds transition to a

clinical infection status and or those wounds that receive therapy but fail. This would present valuable data in being able to identify patterns which suggests shifts towards causing a host immune response that is characterized as clinical infection or those at risk of failing therapy.

In review of the studies employing amplification and sequence analysis of 16S rDNA to characterize the microorganisms involved in DFI, few have sampled participants with overt clinical signs of infection. It is generally regarded that clinically uninfected wounds should not be treated with antimicrobials and therefore should not be cultured [2,94]. The clinical relevance of culturing uninfected and or chronic DFUs to characterize the microbiota is therefore a matter of debate. In patients with diabetes presenting with overt clinical signs of infection, the decision to obtain a culture from a wound to identify a pathogen of infection is clinically justified. It has been well addressed in the literature however, that many patients with diabetes, often do not display overt clinical signs of infection in the presence of a non-healing DFU [19].

Some clinical researchers have proposed under these circumstances to utilize secondary signs and symptoms (*i.e.*, malodor, increased exudate, poor quality tissue) to diagnose infections, that may be more pertinent to guiding clinicians facing wounds that are non-healing in the presence of standard care [2]. Therefore, obtaining samples to examine the microbiota of patients with 'masked' signs of infection in DFUs that are non-healing despite standard care, may prove greatly beneficial.

With the exception of Gardner *et al* (2013) [10] who restricted sampling to a homogenous group of DFUs (neuropathic), all other microbiota studies have included the sampling

of different DFU etiologies. The extent to which community diversity is affected through variances in wound etiologies is currently the focus of debate. It is generally accepted that DFUs of ischemic origin may contain a wound bed environment of devitalized, slough tissue. This may perpetuate very different bacteria to healthy vascularized tissue common in neuropathic DFUs [2,16,95] . Further studies comparing each DFU etiology individually may clarify this matter.

Lastly, it has been cited in the literature that certain aerobic species are often overestimated as the primary pathogens in DFI [42,43]. When comparing the evidence on the bacteriology of DFIs through both culture-dependent and -independent approaches, both corroborate *S. aureus* as being the predominant pathogen colonizing DFUs. In tandem with this, reports have also suggested that the abundance and role of anaerobes are greatly underestimated [10,76]. The significance however at a clinical level, of identifying additional "hidden" anaerobes that often form part of a polymicrobial infection are yet undefined. Many antimicrobial agents commonly used in the treatment of DFIs have a broad-spectrum of activity to cover most anaerobes [96]. Furthermore, no published studies are available reporting improved clinical outcomes when adding additional anti-anaerobic directed therapy to concurrent regimes.

1.3.4. APPLYING METAGENOMICS TO DFI – COULD THIS BE A 'GAME CHANGER' IN DEFINING "WHICH MICROORGANISMS ARE PRESENT, WHAT ARE THEY CAPABLE OF DOING AND "WHO DID IT"

Advancements in molecular technologies have seen the emergence of next generation DNA sequencing platforms (NGS) that greatly increase the throughput of sequencing large amounts of DNA. This significantly reduces the cost to analyse clinical samples and makes NGS platforms widely applicable to use in clinical practice [97]. In tandem with advancements in NGS platforms, has been the output of increasingly larger and more complex data sets. Bioinformatics programs and software packages to cater for this have therefore become an essential tool to complement MPS platforms. Open-source software such as QIIME [98] and Mothur [99] for example, have been widely used in microbiome research for their ability to construct details on three areas of importance to clinicians dealing with chronic wounds: the microbial load, the microbial diversity and the presence of pathogens [38].

Unlike the targeted amplification and sequence analysis of 16S rDNA through PCR, whole genome shotgun sequencing (WGS) (metagenomics) employs a complement of molecular technologies and techniques to sequence DNA extracted directly from a sample. For example, DNA can be sheared into small fragments and independently sequenced in a technique commonly referred to as whole genome shotgun sequencing (WGS). This approach produces sizeable data sets requiring the alignment of DNA reads to known genes through open-access reference databases, such as NCBI GenBank [100]. When combined, these ever-growing gene reference libraries enable the analysis of varying components of microbial ecology and their functions.

Applying metagenomics to DFI may allow for an extended picture of which microorganisms are there. What are they capable of doing and "who" did it? Additionally, metagenomics could provide information on how we should direct therapy. Metagenomics identifies all genomic DNA including bacteria, fungi, archaea and viruses (microbiota) allowing for greater taxonomic (species level) resolution. In addition to DNA sequences involving the taxonomically informative genes (such as 16S rDNA), other genes of interest maybe identified through metagenomics such as protein coding sequences from which the biological functions of the microbe can be inferred *e.g.*, pathogenicity (pathogenicity islands), virulence, antibiotic susceptibility [101] or metabolic pathways [102].

These protein sequences are identified through databases containing resources required for understanding high-level functions and utilities of biological systems, including known proteins for microbial cells (Kyoto Encyclopedia of Genes and Genomes – Kegg and MG-RAST). Metagenomics is however limited in its capacity to provide information on the actual metabolic activity of a microbial community, as it cannot differentiate between expressed and non-expressed genes [103].

To circumvent this limitation, metagenomic methods have combined newer sub-disciplines that utilize mass spectrometry in combination with molecular approaches. Transcriptomics and proteomics can identify expressed biological signatures such as RNA-based regulation or proteins, respectively, that control metabolic activities in microbial communities [104,105]. In this respect, combining metagenomics with transcriptome or proteome analysis may afford the possibility to characterize not only the microbial diversity within a DFI (*i.e.*, "which microorganisms are present") but the

functional potential *i.e.*, "which microorganisms are present and what are they capable of doing and who did it" [102].

Thus, it would be highly desirable to determine which microbes are the potential "assailants", "co-conspirators" and "bystanders"; this would allow for targeted antimicrobial therapy. One study exemplifying the combined use of 16S rDNA sequencing with proteomic analysis was by Lassek *et al* (2015) [106] who elucidated pathogen-protein expressions of catheter-biofilm associated urinary tracts infections. Both *P. aeruginosa* and *M. morganii* were identified as the predominant microorganisms through WGS community analysis. Proteomic analysis then revealed several interesting findings at a functional level which implicated *P. aeruginosa* as the primary driving pathogen and identified that it up-regulated proteins involved in the degradation of red blood cells, the siderophore systems for iron acquisition (i.e. bacterial growth), biofilm formation, antibiotic resistance and in pathogenicity. Currently, no studies employing 16S rDNA sequencing in combination with transcriptome, proteome or metabolome data have been undertaken for DFI.

1.3.5. LIMITATIONS OF DIFFERENT MOLECULAR TECHNOLOGIES AND TECHNIQUES

PCR amplification of DNA fragments requires the use of primers to bind to a specific region of the 16S rDNA enough to produce a PCR product. The use of some universal primers may not be broad enough to detect the bacteria that often cause polymicrobial infections in chronic DFUs [2]. In this respect the microbiota may be overrepresented by certain taxa due to primer bias [101]. Although the hypervariable regions of the 16S rDNA

are highly diverse, this variation is reduced between closely related taxa. Thus, some workflows that use a single gene assay (through 16S rDNA gene sequencing analysis) as the sole sequencing method for community profiling may lead to inconclusive results with closely related taxa [64] or an inability to classify taxa to species level [75]. This limitation can be somewhat partially offset using multiple primer sets (multiplexing PCR) to amplify different hypervariable regions. Lastly, current DNA-based methods are unable to distinguish between live or dead bacteria, amplifying all DNA regardless.

Metagenomics also possess inherent limitations; in particular, the gargantuan and complex data sets it produces with some MPS platforms generating up to 600 Gb of sequence data when performing sequencing runs [107]. Furthermore, both human-host DNA and microbial DNA are sequenced with approximately 90% of DNA from a sample being human.[101] Therefore obtaining adequate coverage of microbial DNA requires deep sequencing (*i.e.*, large data sets) with removal of human DNA sequences via computational resources. Currently, no studies employing metagenomics with or without transcriptome or proteome analysis have been used to determine both microbial diversity and functional potential of samples from patients with DFI. High costs and computational requirements to analyse the complex data may account for this.

1.3.6. SUMMARY

Amplification and sequence analysis of 16S rDNA for profiling the microbiota of infected and non-infected DFUs have already provided insights for the medical community by identifying that many more bacteria are present in DFUs than the corresponding view from culture-dependent methods [10]. Despite the wealth of

knowledge gained from molecular microbiology, the use of 16S rDNA based approaches have not been routinely employed in frontline clinical microbiology services for the identification of pathogens of wound infection. This limited uptake has been attributed to the low throughput and relatively high sample sequencing costs using PCR-based platforms. This may account for why it has been primarily confined as a researcher's tool for characterizing the microbial profile of various infective pathologies in human health and disease.

Whilst the progress and application of current DNA sequencing continues to rapidly evolve, thus far we have only been enlightened with a broader view of "which microorganisms are there" while the interpretation and any clinical implications of additional bacteria within samples remains unclear. The potential of molecular methods that employ metagenomics, transcriptomics, proteomics and metabolomics, represent a potential future 'game changer' to improving clinical care in people with DFI. Their usage may help to reveal the full extent of DFI; "which microorganisms are present, what are they capable of doing and which microorganism/s are responsible.

CHAPTER 2

2.1. MICROBIAL BIOFILMS

Bacteria are often viewed as being single cells that rapidly multiply when in exponential growth and are susceptible to antibiotics if not inherently resistant. Antimicrobial resistance and multi-drug resistance are an increasing problem across the globe and are a current hot topic subject to much debate [108]. Most clinicians involved in the treatment of wounds will utilize susceptibility patterns they receive from the clinical microbiology laboratory, as a guide to determine which antibiotic(s) a patient requires [27].

These decisions are often aided by international consensus guidelines, which are sufficient when managing acute infections [2,25]. However, in cases of chronic infection, such as those seen for implantable medical devices, pulmonary infections of cystic fibrosis patients and chronic non-healing wounds, these guidelines may be inadequate [109]. Why is this? How can we explain the quick resolution of infective symptoms using antimicrobials in patients with acute wounds [110], in comparison to the prolonged response often noted in non-healing chronic wounds [111]?

The answer is both complicated but also rather simple. Bacteria can exist in two (maybe more?) phenotypic different growth forms, that being single fast-growing cells i.e. the planktonic form, and the second as aggregated communities of slow growing cells in a biofilm form. All classical microbiology and development of antimicrobials have been solely based on planktonic paradigms, through methods developed in the early 1800's.

These methods are considerably easier to grow bacteria, through shaken cultures or by spreading on an agar plate, and it is how bacteria presumably exist during acute infections.

These methods are still widely accepted as gold standard for depicting the pathogens of acute infections. The picture for chronic infections is completely the opposite. In this case, a substantial amount of the bacteria resides in biofilms, where they are surrounded by a dense matrix of polysaccharides, free bacterial DNA and proteins that tightly attaches the biofilm community to themselves and structures and protects them from being engulfed and killed by neutrophils and macrophages [112]. In addition, many of the bacteria are not rapidly multiplying or metabolising, which causes the bacteria to become tolerant. This is because almost all antibiotics only work on metabolically active bacteria via their numerous bacterial targets [113,114].

Biofilms are frequently defined based on *in vitro* observations [115]. Classic definitions often describe biofilms as bacteria attached to surfaces, encapsulated in a self-produced extracellular matrix and tolerant to antimicrobials [116,117]. In addition, biofilm development is often described as a three to five stage scenario, beginning with single cells attaching to a surface, maturation of the biofilm and lastly dispersal of bacteria from the biofilm [118,119]. However, *in vitro* observations based on flow cell models utilizing glass surfaces, and fresh oxygenated culture media continuously flowing over the bacterium, differ greatly from the conditions within chronic wound infections [120]. Here, the bacteria are not exposed to a continuous flow of fresh media and are not attached to a glass surface (They adhere to human cells). *In vivo* chronic wound

biofilms are also often encapsulated in matrix that includes host material making dispersal a problematic event [121].

Therefore, using *in vitro* observations to define, diagnose and treat biofilms in chronic infections, may provide a misguided impression [122]. Commonalities between *in vitro* and *in vivo* evidence that we can be more abreast to in providing a definition of a biofilm are; the aggregation of bacteria, some sort of matrix that is not restricted to self-produced since it can also be of host origin, and the tolerance and protection against most antimicrobials and the host defense. There are, however, commonalities between *in vitro* and *in vivo* evidence that can help in providing a definition of a biofilm [93]. These include:

- Aggregation of bacteria
- Some sort of matrix that is not restricted to self-produce as it can also be of host origin
- Extreme tolerance and protection against most antimicrobial agents and the host defence.

How do biofilm communities differ from planktonic bacteria?

Planktonic bacteria are single cells that are usually fast growing and are commonly observed in acute infections [123]. During acute infections bacteria are of the planktonic phenotype, since they are susceptible to antimicrobial agents with targeted treatments causing an abrupt resolution of symptoms.

In vivo evidence has suggested biofilm phenotypes differ markedly in both their physiology and activity when compared with planktonic cells [120]. The bacteria are

aggregated and difficult to treat, if not impossible, somehow evading host defences [93]. Often the bacteria are embedded in a matrix which can be produced by the bacteria or is of host origin. The exact composition of EPS varies according to the microorganism's present, but generally comprise polysaccharides, proteins, glycolipids and extracellular DNA [124-126]. Microelectrode studies have further identified anoxic regions within a biofilm, resulting in lower bacterial cell metabolic activity [127-129]. This contributes in part to the inherent resilience of biofilms to antimicrobial treatments.

2.2. THE PREVALENCE OF BIOFILMS IN CHRONIC NON-HEALING WOUNDS

APPENDIX 5. **MALONE, M.,** BJARNSHOLT, T., MCBAIN, A. J., JAMES, G. A., STOODLEY, P., LEAPER, D., TACHI, M., SCHULTZ, G., SWANSON, T., WOLCOTT, R. D. THE PREVALENCE OF BIOFILMS IN CHRONIC WOUNDS: A SYSTEMATIC REVIEW AND META-ANALYSIS OF PUBLISHED DATA. JOURNAL OF WOUND CARE 2017. 26:1, 20-25

Candidate contribution:

- ✓ Worked as primary author for publication undertaking 90% of the manuscript
- ✓ Wrote the manuscript in full using information from thesis chapters 1 and 2
- ✓ Undertook extensive systematic review of the literature
- ✓ Performed meta-analysis statistics
- ✓ Reviewed and amended all required changes from co-author contributions
- ✓ Submitted the manuscript as primary author

Abstract

Evidence supporting the presence of biofilms in chronic non-healing wounds is continuing to advance. A large proportion of what we have learnt about biofilms and how they may contribute to the chronicity of wounds are derived from *in vitro* model and *in vivo* animal data. However, human chronic wound studies are under-represented with most studies having low sample sizes. For this reason, we sought to ascertain the prevalence of biofilms in human chronic wounds by undertaking a systematic review and meta-analysis.

Only studies that used rigorous methods for sample collection (biopsy or curettage) and visualization of biofilm consistent with recent guidelines (light microscopy, scanning or transmission electron microscopy) with or without molecular methods were included. Our initial search identified 554 studies from the literature databases (Cochrane Library, Embase, Med-line). After removal of duplicates, and those not meeting the requirements of inclusion, 9 studies involving 185 chronic wounds met the inclusion criteria. Between-study heterogeneity was high (Q test $p = .022$, I2 = 55%) so a random-effects meta-analysis model was utilised. Pooled visual prevalence of biofilms in chronic wounds was 78.2% ($p = .002$, CI 61.6 – 89). The results of our meta-analysis support our clinical assumptions that biofilms are ubiquitous in human chronic non-healing wounds.

2.2.1. INTRODUCTION

During most of the history and development of microbiology, the general understanding of the role microbes play in human health and disease has been that they exist as

planktonic or free-floating single cell organisms. Seminal works by Louis Pasteur and Robert Koch in the mid to late 1800s paved the way in the field of microbiology and laboratories still use the 150-year old methods developed by these pioneers. These techniques postulate that microbial cells act in a planktonic state, that is, they disperse in a liquid environment.

However, emerging evidence from the previous century, based on microbial studies of aquatic environments and dental plaque provided insights that microorganisms have a natural tendency to associate surfaces, preferring a sessile lifestyle [130,131]. This early work, which focussed predominantly on environmental samples, later provided a platform for the contemporary medical models that we have come to understand as "microbial biofilms". Unlike their planktonic counterparts, biofilm phenotypes have been defined as structured consortiums of aggregated microbial cells, surrounded by a polymer matrix, that adhere to natural surfaces or to themselves [132].

The concept of biofilms in human health and disease is now universally accepted in tuberculosis [133], periodontal disease and dental caries [134], cystic fibrosis [135-137], in-dwelling medical device infections [138], otitis media and other upper respiratory infections [139,140], and chronic wounds [12,90,141]. So highly attuned are researchers to the wide involvement of biofilm associated infections across the spectrum of human health and disease, the United States Department of Defence for example, has recognized the significance of biofilms as being problematic in wound healing, and has prioritized research in this area [142].

Unlike some commensal sessile microbial communities, microorganisms residing within a non-healing chronic wound in the biofilm phenotype may promote a hyper-inflammatory response as a persisting adverse pathology, much to the detriment of the host [8,143,144]. Recent observations have also provided alternative insights into how bacterial biofilms in chronic wounds may promote chronicity. Using oxygen microsensors and transcriptomics (examining microbial metabolic activities), James and colleagues identified steep oxygen gradients within in situ biofilms and induced oxygen-stress responses from bacteria [129].

Once established, biofilms often become highly tolerant to standard treatment and removal/eradication paradigms, yielding several hallmark features that distinguish biofilm phenotypes from their planktonic counterparts. The most notable of these is a remarkable tolerance to antimicrobial agents [145], disinfectants and host immune defenses [146,147].

Whilst non-healing chronic wounds represent an umbrella terminology for a range of pathologies, biofilms have been cited across all related etiologies including;- venous leg ulcers (VLU's) [148], pressure Injuries (PI) [143,149] and diabetic foot ulcers (DFUs) [12]. Collectively these chronic wounds contribute to significant morbidity, mortality and increased healthcare expenditure. Importantly, the continuing rise in antimicrobial resistance has placed a greater emphasis on correctly diagnosing and managing biofilm associated infections in non-healing chronic wounds. This will require a shift in treatment paradigms to more multifaceted biofilm-based approaches given the resilience of biofilms in responding to planktonic-based treatments.

As the presence of biofilms across the spectrum of chronic wounds has significant implications both medically and economically, clear and concise information is required to help guide healthcare professionals managing these recalcitrant causes of delayed healing. Over the last decade an increasing body of evidence from *in vitro* models and animal [150,151] and human studies has identified the capacity of wound isolates to grow as biofilms, and for chronic non-healing wound samples to harbour biofilm. This has been driven largely by advancements in molecular microbiology and microscopy technology and techniques applicable to the study of bacterial populations in situ. This has allowed authors to implicate biofilms as the cause of non-healing chronic wounds and in the development of associated clinical infections.

There is a plethora of *in vitro* model and *in vivo* animal data supporting the presence of biofilms in non-healing chronic wounds [152-155]. A recent review of the scientific literature for the presence of biofilms in chronic wounds has eloquently explored the models utilised[156]. However, human chronic wound studies are under-represented with most studies having low sample sizes. For this reason, we proposed to ascertain the prevalence of biofilms recognised in human chronic wounds by systematically reviewing the literature from published *in vivo* human chronic wound studies to increase sample size and power to provide a meta-analysis.

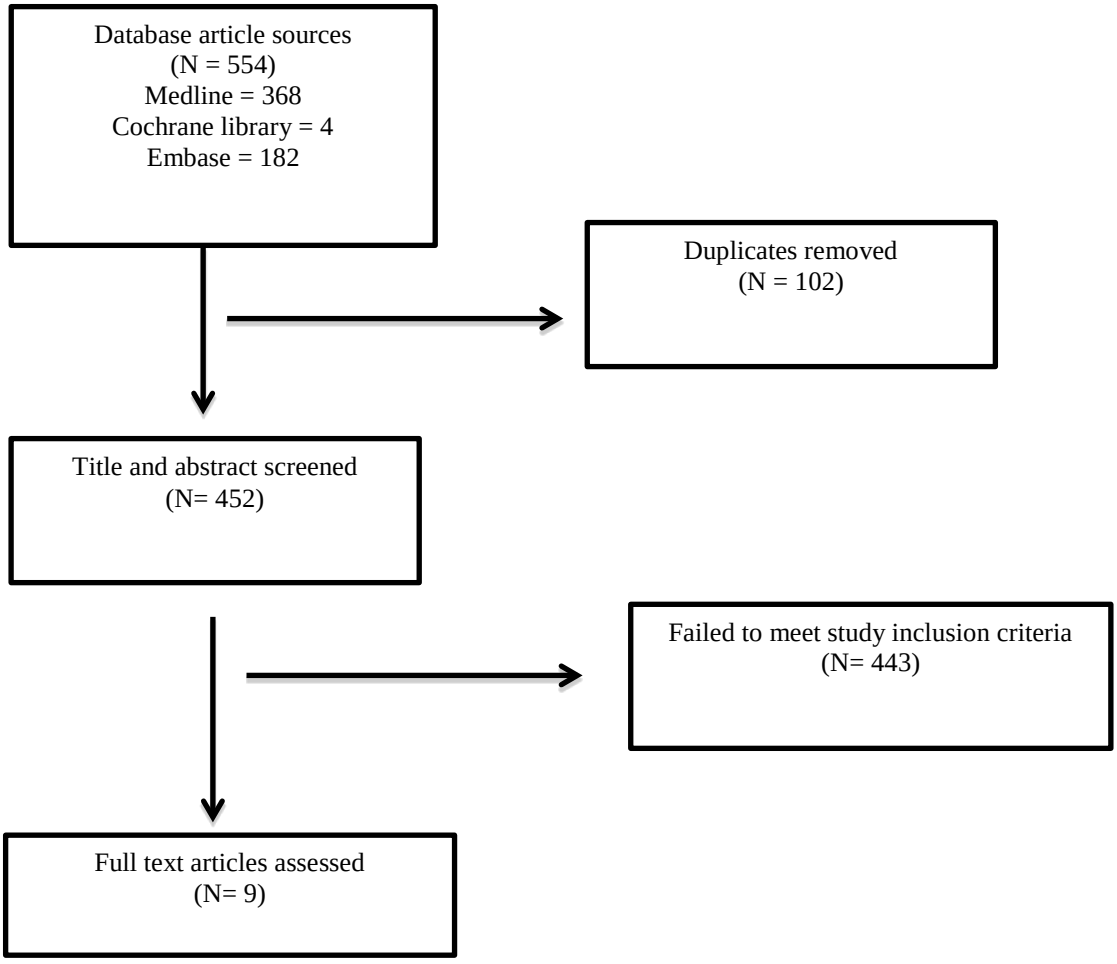
2.2.2. METHODS

Search strategy

An electronic search of the literature was performed to identify published studies on the broad area of biofilms in chronic wounds with the primary aim to ascertain the percentage of chronic wound samples that contain biofilm. A systematic review of the

Cochrane Library, Embase, Med-line (PubMed) databases was conducted between January 2008 and December 2015 using the following search term “biofilm” [all fields] AND “chronic wounds”. A secondary search was also undertaken using ‘biofilm” with supplementary keyword filters; OR “diabetic foot ulcers” OR “venous leg ulcers” OR “pressure ulcers” OR “decubitus ulcers” OR “non-healing surgical wounds”, OR “visualization”, OR “scanning electron microscopy” OR “fluorescent in situ hybridization”, OR “16S rRNA”. Only articles limited to English language were included. The search was limited to prospective clinical studies, case reports, case series and published conference abstracts. The systematic review was conducted in accordance with the PRISMA guidelines (Figure 2.1) [157].

Figure 2.1 PRISMA flow diagram of literature search.



Data extraction

Two investigators (MM and TB) independently reviewed titles and abstracts of all articles to establish their eligibility based on predefined criteria. All eligible article references were tabled, and their abstracts obtained for review. Articles meeting the eligibility criteria were hand-searched for additional studies. For the meta-analysis, we extracted the following domains or variables from the articles that included, date of study publication (2008 – 2015), prevalence rates (number of confirmed tissue samples over the total number of samples screened), sample size and study design.

Study eligibility

Articles publishing data on *in vivo* human chronic wounds, in participants over the age of 18 were included. Chronic wound aetiologies included in the search were diabetic foot ulcers (DFUs), venous leg ulcers (VLUs), pressure injuries/ulcers (PI/PUs) and non-healing surgical wounds (NHSW). Individual searches of the methodology section from each paper were undertaken and universal definitions of a chronic wound or phrases denoting the chronicity of participant wounds such as “non-healing”, “delayed healing” and or “chronic” were used to ensure eligibility.

Only articles detailing the presence of biofilms and bacteria in general through microscopy with or without combined molecular methods were included for review. In line with recent guidelines[93] the following visualization techniques were deemed appropriate for the confirmation of biofilm presence; scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM), conventional and peptide nucleic acid - fluorescent in situ hybridisation (PNA-FISH) and microscopy with or without staining methods. Articles diagnosing biofilm presence by clinical observation were excluded.

Additionally, to meet inclusion, articles must have cited optimal collection methods for the sampling of chronic wounds with tissue biopsy, curettage or debridement material being regarded as the preferred sampling method. Swab cultures of the wound bed were excluded for being inadequate for biofilm identification, given the inability to detect between planktonic and biofilm phenotype[93].

Statistical analysis

Data from studies were extracted as raw numbers using the number of samples with confirmed biofilm over the total number of samples obtained. Data were analysed using comprehensive meta-analysis software (Biostat Inc., New Jersey, United States). Pooled prevalence estimate rates, weighted averages and 95 % confidence intervals (CIs) were undertaken using fixed-effects meta-analysis. Forest plots were reported for inconsistencies in effect sizes and their confidence intervals. Between-study variance or heterogeneity in estimates was modelled using Cochran's Q and the I2 statistic. Where Cochran's Q value was reported with p-values < 0.10 and I2 values exceeded >50%, a random-effects model was used [158].

2.2.3. RESULTS

The search identified 554 studies from the literature databases. After removal of duplicates, exclusion and the screening of 452 titles and abstracts, eight studies involving 185 chronic wounds met the inclusion criteria (Figure 2.1). The numbers of each respective chronic wound were; DFUs (n = 33), VLUs (n = 67), PI (n = 26), NHSW (n = 28), Unspecified chronic wounds (n = 31). Eight articles were from prospective cohort studies with the remaining one study being case reports / series (Table 2.1). Primary authors were contacted for data from two studies to clarify the number of positive biofilm samples [159,160]. As expected, between-study

heterogeneity was high (Q test P< 0.022, I2 = 55%). To address this, a random-effects model was utilised with pooled prevalence rates reported.

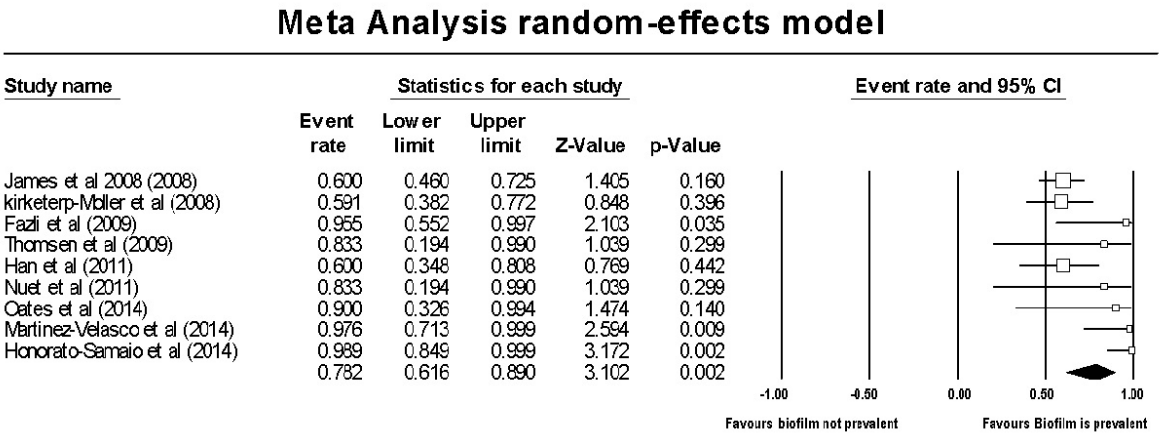
Author/s	Study design	Number of participants	Visualisation methods with or without molecular	Chronic wound aetiologies	Nº of samples with confirmed biofilm (%)
James *et al* (2008) [12]	Prospective study case vs control	66	Light microscopy, SEM 16S rDNA with DGGE	13 DFUs, 21 PUs 8 VLUs, 24 NHSW	30 out of 50 (60%)
Kirketerp-Moller (2008) [13]	Prospective cohort study	22	PNA-FISH, CLSM	Un-specified chronic wounds	13 of 22 (60%)
Fazli *et al* (2009) [161]	Prospective cohort study	9	PNA-FISH, CLSM	10 VLUs	10 of 10 (100%)
Thomsen *et al* (2009) [39]	Prospective cohort study Sub analysis	2	PNA-FISH, 16S rDNA	2 VLUs	2 of 2 (100%)
Han *et al* (2011) [78]	Prospective cohort study	15	PNA-FISH, CLSM 16S rDNA	4 DFUs, 5 PUs, 2 VLUs 4 NHSW	9 of 15 (60%)
Neut *et al* (2011) [162]	Case report	2	CLSM	2 DFUs	2 of 2 (100%)
Oates *et al* (2014) [163]	Prospective cohort study Sub analysis	4	FISH, SEM,	4 DFUs	4 of 4 (100%)
Martinez-Velasco *et al* (2014) [159]	Prospective cohort study conference abstract	20	SEM, LM	Un-specified chronic wounds	20 of 20 (100%)
Honorato-Sampaio *et al* (2014) [160]	Prospective cohort study	45	TEM	45 VLUs	45 of 45 (100%)

Table 2.1 Descriptions of included studies utilising microscopy approaches to visualise wound biofilm. Scanning Electron Microscopy (SEM), Light microscopy (LM), partial nucleic acid fluorescent in situ hybridisation (PNA-FISH), confocal laser scanning microscopy (CLSM), denaturing gradient gel electrophoresis (DGGE), amplification and sequence analysis of the 16S rRNA gene (16S rDNA), diabetic foot ulcer (DFU), venous leg ulcer (VLU), pressure ulcer (PU), non-healing surgical wounds (NHSW).

Prevalence of biofilms in chronic wounds

The pooled prevalence of biofilms in chronic wounds was 78.2% (p = .002, CI 61.6 – 89) (Table 3). Biofilm prevalence varied greatly over all studies, however the percentage(s) of positive biofilm samples was no lower than 60% noted in three studies [12,13,78], with all remaining studies identifying 100% biofilm prevalence [159-164]. Given the relatively small sample size and the co-variable of 4 different chronic wound aetiologies, inferences regarding whether biofilms were more prevalent in one chronic wound were not possible.

Table 2.2 Random–effects model of nine chronic wound biofilm studies.



2.3.4. DISCUSSION

Early landmark publications providing evidence for the presence of biofilms in chronic wounds have provided guidance for clinicians and researchers alike [12,13,141]. These studies identified that biofilms were present in 60% of non-healing chronic wounds. Since then, studies employing combined molecular and microscopy methods to directly visualise biofilms have gathered pace.

This systematic review and meta-analysis is the first to collate all available *in vivo* studies pertaining to the identification of biofilms from non-healing human chronic wounds. In doing so, our meta-analysis results suggest that biofilms are prevalent in all these wounds. Pooled prevalence rates identify that 78% of non-healing chronic wounds harbour biofilms, with prevalence rates varying between 60% and 100%. We propose therefore, that biofilms are ubiquitous in nearly all non-healing chronic wounds and the disparity in prevalence rates maybe a reflection in study design and methodological limitations. For example, we argue that heterogeneous distribution of microorganisms within wounds may allow for variability in sampling, increasing the likelihood of returning negative or inconclusive samples.

Three previous studies [13,39,40] have highlighted the heterogeneous spatial distribution of wound microbiota through sampling multiple areas of the wound bed, identifying vast shifts in community diversity. This suggests relying on a single site for sampling may reduce the chances of visualizing biofilm. Obtaining samples from multiple sites of the wound may improve the detection of biofilm. However, this is often not feasible at a clinical level and is reflected in many studies that employ tissue collection methods.

When combining the results of our systematic review and meta-analysis with the plethora of *in vitro* models and *in vivo* animal studies it seems highly likely that biofilms are ubiquitous in non-healing human chronic wounds. It is important therefore, that clinicians appreciate the distinct differences of biofilm phenotypes to their planktonic counterparts, the challenges in eradicating and removing biofilm from a wound. A paradigm shift to a biofilm based wound care approach should be adopted.

2.3. SIGNIFICANCE OF MICROBIAL BIOFILMS IN CHRONIC WOUNDS

2.3.1. CLINICAL SIGNS AND SYMPTOMS OF BIOFILMS IN CHRONIC WOUNDS

Current methods to visualise biofilm from tissue samples have been confined primarily to use by researchers of high-powered microscopes (scanning electron microscopy – SEM) or in combination with molecular DNA sequencing techniques that utilise fluorescent probes to determine the presence or absence and location of bacteria (Confocal laser scanning microscopy - CLSM). Given then, biofilms are microscopic by nature; this has led to a grumbling argument over whether biofilms can be visually observed by clinicians.

Under certain circumstances, biofilms when left to thrive may show evidence at a macroscopic level, with one example being oral plaque [134]. The picture however is less clear for chronic wounds. Some clinicians have used rhetoric to promote what they believe are “clinical cues” of biofilm presence, through naked eye observations not based on scientific rigour [165-168]. Such signs have included a shiny, translucent, slime layer on the non-healing wound surface, the presence of slough or fibrin, and gelatinous material that reforms quickly following removal in contrast to slough and other devitalised tissue, or fibrin that often takes longer to reform.

Assumptions to suggest biofilm involvement in non-healing chronic wounds or associated infection have used observations on the characteristics of disease progression. These have included contrasting symptomology, where acute infections instigated by planktonic microorganisms exhibit rapid onset symptoms. In contrast, biofilm related infections have been acknowledged for their lower acuity and longevity, often persisting with transient symptoms for months or even years [169].

Observations of patients receiving oral antimicrobial therapy for biofilm-associated chronic infection have highlighted periods of quiescence, that alternate with acute exacerbations once antimicrobials are discontinued [170,171]. Once antimicrobial therapy ceases, the biofilm serves as a nidus for recurrence of infection with acute exacerbations again manifesting [145]. The transient suppression of microbial cells demonstrates the tolerance of biofilms to antimicrobials and standard treatment regimens. The suppression of infective symptoms may occur through bactericidal action against planktonic microbes shed from the biofilm, or action against the most superficial of biofilm cells [172]. Failure to eradicate dormant microorganisms within the deeper stratum of biofilm, have also been suggested as a mechanism for reconstitution [172].

Presently, there is no gold standard diagnostic test to define the presence of wound biofilm and there exist no quantifiable biomarkers. This may pose significant implications at a clinical level, where the inability to distinguish between planktonic or biofilm-based pathogenicity may lead to ineffective treatment.

2.3.2. HOW DO BIOFILMS IMPEDE WOUND HEALING?

The exact mechanisms of biofilm impairment on the healing processes of wounds remain ambiguous. Existing data suggest a chronic wound is kept in either a severe inflammatory state, or subject to localized low oxygen tensions. The pathways behind this are not clear, but several systemic and local factors may contribute to the occurrence and maintenance of the wound chronicity.

2.3.3. BIOFILMS MAY SUSTAIN HYPER-INFLAMMATION

In a review article, Wolcott *et al* (2008) [144] present a detailed hypothesis summarising that once a biofilm community becomes established, their stubbornness and often failure to many treatments propagates hyper-inflammation. Specifically, they propose that a biofilm "hijacks the host immune response" through various general non-specific pathways to create a hyper-inflammatory state within the chronic wound.

Wolcott *et al* (2008) hypothesise that biofilm phenotype bacteria produce proteases that inhibit and destroy extracellular matrix. In addition, the chronic wound environment also possess host derived proteases. Together this may over-fill a chronic wound with a proteolytic mix of proteases, elastases and gelatinases (these are commonly referred to as matrix metalloproteinases - MMPs). In parallel, biofilm adherence to the wound bed may also potentially inhibit the release of the natural suppressor of MMPs – tissue inhibitors of MMPs (TIMPS). This scenario may therefore sustain a perpetual state of hyper-inflammation.

Another mechanism to sustain a hyper-inflammatory state proposed by Wolcott *et al* (2008) is the manipulation of neutrophil function. The over-abundance of neutrophils within chronic wounds is well documented [173,174]. A profound neutrophil response is both normal and required

for bacterial eradication following disruption to the skin barrier, however excessive neutrophils are detrimental to wound healing [173,174]. Wolcott *et al* (2008) suggest neutrophils are over-expressed through biofilm stimulation of IL-8, the chemo-attractant cytokine acting on neutrophils [175]. No *in vivo* data on human chronic wounds exist to support this hypothesis.

Wolcott *et al* (2008) also put forward the potential of biofilms to 'bait' the immune system through releasing planktonic microorganisms. Thus, biofilm survival is maintained through planktonic seeds released so they can bait the immune system through the PAMP recognition pathways, inducing an inflammatory response and nutrient pathway. The resulting exudate and its nutrients could then be utilised for biofilm growth. Wolcott *et al* (2008) also suggests an increased role for anaerobic microorganisms who may play a key role in biofilms sustaining a hyper-inflammatory host response, by releasing lipopolysaccharide, a potent inflammatory inducer. In an animal model on cystic fibrosis patients, biofilm forming *P. aeruginosa* were shown to undergo lipopolysaccharide modifications that induced greater inflammatory responses in mice [176]. No human *in vivo* data exist to support this theory for chronic wounds.

2.3.4. BIOFILMS MAY CONTRIBUTE TO LOCALIZED AREAS OF LOW OXYGEN TENSION WITHIN A WOUND.

Early microelectrode studies of aerobic *in vitro* biofilm models found discrete areas within biofilm that had significant oxygen depletion [177]. These suggested areas of biofilm, housed micro-niches favouring differing microorganisms, and may explain how the presence of anaerobes in mixed-species biofilms, exist, contribute and cooperate with aerobic neighbours. Further studies employing microelectrodes with CLSM have identified micro-domains with different biochemical environments including alterations in pH and oxygen [178]. Recent data by

James *et al* (2016) [129] has provided further evidence to support a concept of a localized low oxygen tensions contributing to wound chronicity. Using oxygen microsensors and transcriptomics (examining microbial metabolic activities) to study in situ biofilms, James and colleagues identified steep oxygen gradients and induced oxygen-limitation stress responses from bacteria. James *et al* (2016) identified through transcriptomics, that inferred metabolic activities of the biofilm and the recruitment of cells that consume oxygen for host defensive processes, were the primary pathways of oxygen depletion. Taken collectively, this data supports the concept of a biofilm establishing and maintaining localized low oxygen tensions in a wound could potentially contribute to wound chronicity.

2.3.5. THE EFFECT OF BIOFILMS ON THE HOST-MICROBE INTERACTION AND HOW THEY MAY IMPEDE WOUND HEALING.

Data on the interactions of the host - biofilm phenotype have been primarily undertaken using single species biofilm models of *S. aureus* and *P. aeruginosa. In vitro* data presented by Tankersley *et al* (2014) [179], has revealed *S. aureus* biofilms may have a direct impact on human epithelial keratinocytes. The effects of *S. aureus* biofilms and planktonic *S. aureus,* on gene expression and up-regulation of inflammatory cytokines by human keratinocytes, were explored. *S. aureus* biofilm induced significant alterations in gene expression from human keratinocytes in comparison to planktonic *S. aureus*. Forty-two inflammatory associated genes were over-expressed, including IL-8, IL-6 and Nitric Oxide (p = .05). Keratinocytes have several key roles in wound healing, and the ability to induce numerous cellular pathways makes them important as both the first line of defence and in the re-epithelialization of wounds [180]. If a biofilm can induce an effect to cause up-regulation of pro-inflammatory cytokines capable of degrading the extracellular matrix of wounds, this may directly impact re-epithelialisation [181].

Schierle *et al* (2009) [182] used an *in vivo* mouse model to examine *S. aureus* biofilm involvement in preventing wound healing. Full-thickness excisional punch wounds were made into six adult male C57B16/J mice and inoculated with *S. aureus* (strain CFS101), with six controls free from inoculation. Biofilm formation was determined post 3 days using microscopy and fluorescent stains. All six inoculated mice demonstrated gross evidence of biofilm formation. This correlated to a delay in re-epithelialization at day 9 of wounding, in comparison to control mice where all wounds healed (Epidermal gap: *S. aureus* = 2.4 vs. 0.5mm, $p = 0.01$).

Gurjala *et al* (2011) [183] developed a novel *in vivo* animal model to assess the effects of *S. aureus* biofilms on wound healing. Full thickness dermal punch wounds on the back of New Zealand white rabbit's ears, were inoculated with *S. aureus* (strain UAMS -1). Biofilm presence was confirmed using SEM and CLSM. Biopsy samples identified biofilm growth was associated with reduced granulation tissue formation and delayed epithelial migration.

In a *P. aeruginosa* biofilm model, Zhao *et al* (2013) [184] inoculated five db/db mice with a biofilm producing wild type strain of *P. aeruginosa* (PAO1). Four mice were left un-inoculated as controls and monitored for 28 days. At day 28, PAO1 inoculated mice experienced a significant delay in wound healing with no mouse healing. In comparison, all the control mice had healed within the study time frame. Histological analysis showed extensive inflammatory cell infiltration, tissue necrosis and epidermal hyperplasia adjacent to challenged wounds- all indicators of an inflammatory non-healing wound.

Fazli *et al* (2011) [161] analysed the cellular inflammatory response of tissue obtained from five CVLU samples with confirmed *P. aeruginosa* biofilm, and five CVLU samples containing confirmed *S. aureus* biofilm. Staining methods using haematoxylin and eosin revealed that

PMNs were abundant around biofilms but failed to infiltrate the EPS. In comparison to *S. aureus* biofilm samples, *P. aeruginosa* biofilms had significantly greater PMN accumulation (range 57-96 cells per image *vs* 18-50 cells per image, $p < .0001$). This may suggest that the presence of a *P. aeruginosa* biofilm can increase the cellular inflammatory response through in situ neutrophil accumulation. Alternatively, Jensen *et al* (2006) [185] have identified the capability of *P. aeruginosa* biofilms to rapidly kill PMNs through the production of rhamnolipid. Rhamnolipid, is regulated via quorum sensing communication and its production by the biofilm phenotype of *P. aeruginosa* may serve as a PMN protective force field.

Marano *et al* (2015) [186] identified that migration and proliferation of human epidermal keratinocytes were decreased by derivatives from biofilms of *P. aeruginosa* and *S. aureus*. Employing proteomic analysis, Marano *et al* (2015) mapped *S. aureus* activity to a protein, while *P. aeruginosa* activity was due to a small molecule. The several proteins revealed through proteomic analysis had putative links to delayed wound healing. These included alpha hemolysin, alcohol dehydrogenase, fructose-bisphosphate aldolase, lactate dehydrogenase and epidermal cell differentiation inhibitor.

In vitro models and *in vivo* animal studies of both *S. aureus* and *P. aeruginosa* biofilms and their secretions, identify potential mechanisms of impeding wound healing. Data suggests that both microorganisms possess several complex and potential pathways for inducing wound chronicity. *S. aureus* in a biofilm mode of growth may play a role in altering the up-regulation of pro-inflammatory cytokines through human keratinocytes. Conversely, the sustained in situ necrosis by microbial cells could explain both the constant influx of PMNs into chronic wounds containing *P. aeruginosa*, in addition to the resulting localised releases of proteolytic enzymes that are pro-inflammatory [144].

Wolcott *et al* (2016) [187] undertook a study design in order to ascertain if microbiota removed from a chronic human wound could actively produce a new chronic wound in an animal model. In short, the authors attempted to prove Koch's postulates and show that chronic wound microbiota is fully capable of propagating on new host tissues and is a cause of delayed healing. To prove this concept, Wolcott obtained wound bed material via debridement and transferred this onto wounded Swiss Webster mice. Three mice were used as controls. 16S rDNA sequencing was performed on sections of human wound debridement material to identify the residing microbiota.

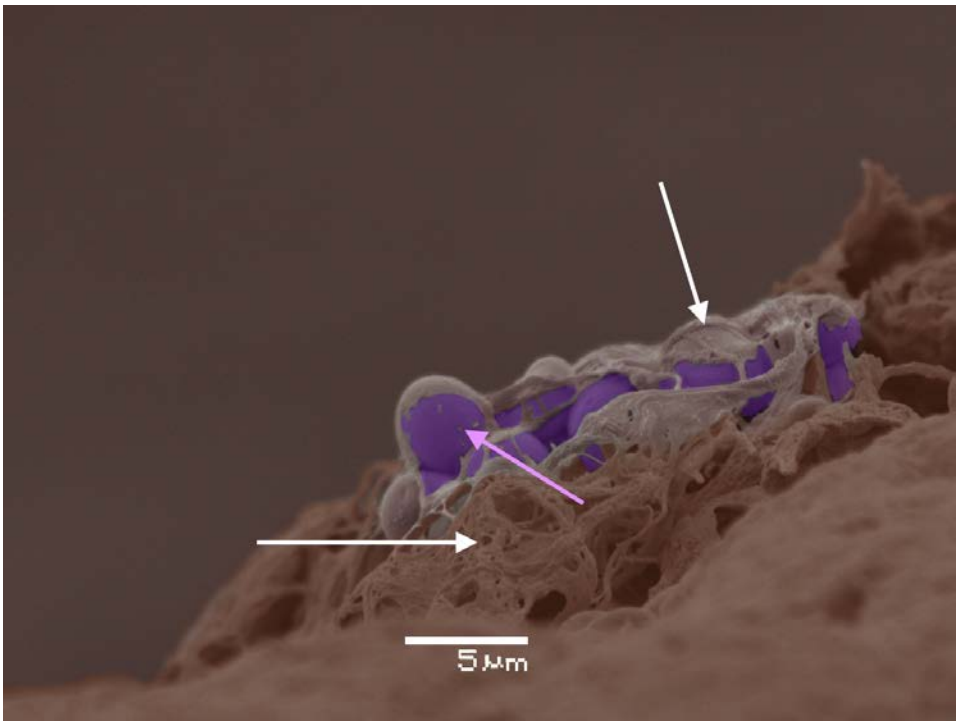
After four days, control mice failed to develop any measurable microbiota as identified through 16S rDNA sequencing. In contrast, mice inoculated with human tissue developed clinical signs of wound chronicity. The symptoms included the development of excessive slough, exudate and slow progression of wound closure. 16S rDNA sequencing also revealed 93% of the measurable bacterial load identified identified in the mouse model were from the human tissue debridement samples. Furthermore, the wound microbiota was not significantly altered through the process of sampling, re-inoculation into a secondary host tissue, and final re-isolation.

2.3.6. EXTRACELLULAR POLYMERIC SUBSTANCE (EPS) IS A THREE-DIMENSIONAL FORCE FIELD THAT ENCLOSES SESSILE BACTERIA.

Costerton and Irvin (1981) [188] first described a process by which bacterial cells produced a 'slime' like substance they termed 'glycocalyx', and proposed it provided additional benefits to microorganisms in a sessile phenotype. The glycocalyx was defined as being a composition of polysaccharides that accounted for over 90% of a biofilm, with less than 10% being bacterial

in composition [124]. The terminology of glycocalyx, was redefined in later years to extracellular polymeric substance (EPS) [189,190], as it became clear that bacterial glycocalyx were more than just polysaccharides. EPS were characterized as biopolymers, composed of proteins, nucleic acids, lipids and humic substances, enabling the immobilization and cohesion of bacterial cells in close proximity [124] (Figure 2.2).

Figure 2.2 Scanning electron microscope of a diabetic foot ulcer identifies bacteria in aggregates (purple) with the production of EPS (white).



The EPS or matrix represents the immediate environment for microbial cells in a biofilm [124,191], producing its own microenvironment by affecting porosity, density, water content, charge, and sorption properties. The exact function, structure and physiochemical interactions of EPS have not been fully depicted owing to the inherent difficulty in analysing EPS in situ [191]. The diversity of residing microorganisms and environmental influences, likely contribute to the final architecture of the EPS, the quantity of EPS production, and its function [126,192]. In some

ways, the term biofilm is a misnomer, as it conjures up thoughts of being a one-dimensional monolayer of slime. In stark contrast, a biofilm is a complex, heterogeneous three-dimensional structure containing dense pockets of bacterial micro-colonies (either monomicrobial or polymicrobial) [117,188,193-196].

In vitro models utilizing CLSM with computational programs have depicted biofilm morphology and architecture [197]. Certain biofilms present as flat structures, whilst some take on mushroom appearances containing channels to funnel nutrients, water and waste products [177]. For example, the most widely documented evidence for EPS production is based on *in vitro* mucoid strains of *P. aeruginosa*. Its production of alginate has profound impacts on biofilm architecture where it can induce a more heterogeneous structure when compared to non-mucoid strains that develop flat homogenous biofilms [198,199]. In tandem, a down regulation of the flagellum of *P. aeruginosa* occurs, suggesting it halts mobility to become a productive biofilm stabiliser [200].

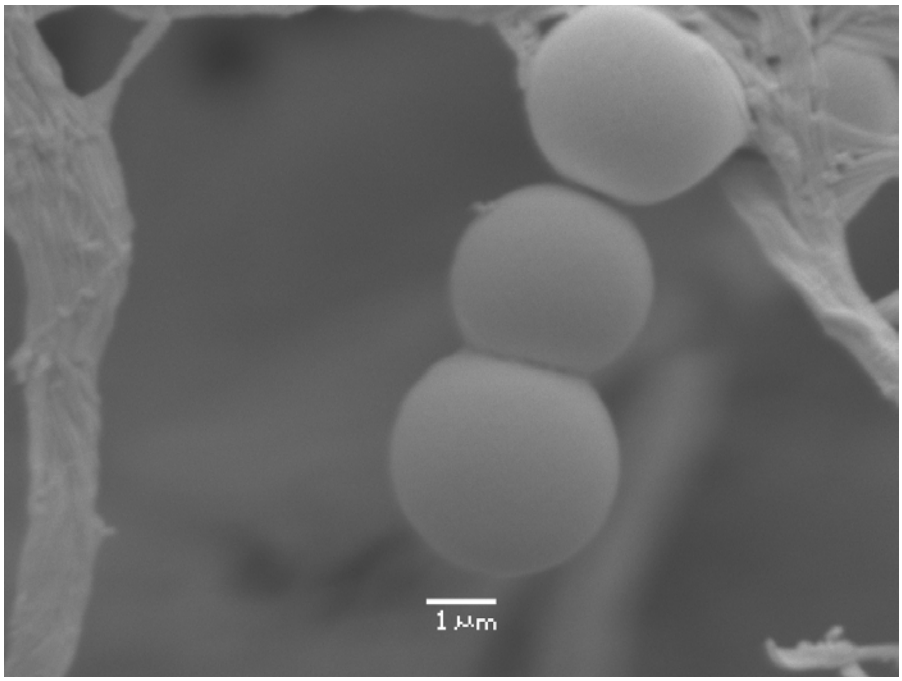
Several functions of EPS have also been elucidated. As a hydrated biopolymer, the EPS interacts with the environment. Through its sorption properties it can sequester substances from the surroundings, thus providing nutrients for microbial cells [201]. The EPS creates the ideal environment to allow for enhanced cell-cell communication and synergism [124]. The benefits of microorganisms remaining in close proximity under biofilm conditions are ideal for promoting higher horizontal gene transfer [202]. This creates a pantry of mobile genetic elements and plasmids that may promote stable social interactions, fitness and survival of usually more vulnerable microorganisms to environmental stresses [202,203]. Perhaps the most discussed function of the EPS has been its role as protective shield against biocides, antimicrobials and the host immune response. This will be explored later in this chapter.

2.4. BIOFILM LIFE CYCLE: FROM ATTACHMENT TO DISPERSAL

2.4.1. BACTERIAL ADHESION

The selectiveness of microorganisms in natural environments to attach to a surface or to each other (Figure 2.3) is so pervasive, that it advocates a strong selective advantage to those choosing a more sessile and social lifestyle [193,204]. Microbial adhesion initiates when microorganisms move into close proximity of a surface (usually <1nm) [205]. Attachment is governed by numerous variables that include, the composition of the surface, the environmental milieu, the bacterial species, available gene products, and communication of microorganisms via quorum sensing [205]. In some environments, an intermediary step prior to primary adhesion may result surface conditioning. This process involves the alteration of a substrate, through physiochemical interactions to improve adhesion conditions [206,207].

Figure 2.3 A tissue biopsy of a diabetic foot ulcer identifies bacterial cell-cell adhesion and cell-surface (collagen) attachment.



Adhesion of microorganisms to a surface is a sequential process initiated through primary adhesion. When a microorganism approaches a surface (or another microbe), a range of mechanical forces (including van der Waals, electrostatic, hydrodynamic and hydrophobic) and or specific molecular anchors aid adhesion (i.e. Adhesin, ligand, EPS) [208-210]. Initially, microbial docking in primary adhesion is undertaken through a reversible bond allowing detachment should conditions be unfavourable [211].

Examples of microbial anchors to aid adhesion include lipopolysaccharide (LPS) found on the outer membranes of Gram-negative bacteria [209]. Gram-negative microorganisms with defects in LPS show reduced adhesion capabilities [212]. Pilus or fimbri are hair-like nano-structures found on Gram-negative bacteria. Their involvement in adhesion and biofilm formation has identified type IV pili, hosting specific binding receptors to target molecules in human hosts [213].

Microbes also host a repertoire of adhesion molecules to target adhesion to human tissue or other abiotic surfaces, such as in-dwelling medical devices. *S. epidermidis* and *S. aureus* possess microbial surface components recognizing adhesive matrix molecules. These molecules bind to proteins such as fibrinogen, fibronectin or collagen [214]. Additionally, *Staphylococcus* spp., have non-covalent bound surface proteins known as autolysins, that have strong affinities for both plastic surfaces and binding receptors of human tissue [215].

The secondary phase of adhesion involves the permanent anchoring of microorganisms to a surface and is governed by global anchors such as EPS [208]. During permanent attachment, planktonic microorganisms may preferentially seek microorganisms of the same species that

have already established themselves in a sessile lifestyle. Planktonic microorganisms may also seek to adhere to different species, where benefits of synergism maybe exploited. Data by Leung *et al* (1996) [216] identified the synergistic effects of both Gram-positive and Gram-negative biofilm microorganisms on plastic stents, and found that colonisation of *Escherichia coli* facilitated the subsequent attachment of *Enterococcus* spp. Ultimately, the outcome of adhesion, whether to a surface or microbe to microbe, is the formation of aggregates and the promotion of differential gene expression for biofilm phenotype.

2.4.2. BIOFILM MATURATION; SHAPING THE MICROBIAL CITY.

Once planktonic microorganisms alter their behaviour through attachment and micro-colony formation to progress into mature biofilms, the residing microorganisms, their interaction with one another, and environmental pressures largely control the maturation process [217]. This social interaction between microorganisms is both an area that has divided researchers, and spawned an entirely unique area of research termed "sociomicrobiology" [218]. The importance of bacterial cooperation versus competition is highly relevant to biofilm mode of growth. Changes to respiratory rate, oxygen consumption, motility and the synthesis of EPS, shape the living structure and composition of a biofilm [65].

In mixed species biofilms, bacterial aggregation and microcolonies do not seem to form by random chance. Instead biofilms form communities with similar requirements and vested interests and settle in microenvironments where survival is possible through the power of cooperation [65,219-221]. Kreft (2004) [222] illustrated the concept of altruism in biofilms using computational modelling to identify fast growth – low yield strains or "selfish individuals" would often outcompete slow growth – high yield strains. When the model introduced single

cell lineage of sloth growth –high yield strains, the out-competing of "cheaters" was possible. Kreft (2004) concluded this was through the promotion of altruism, a fundamental requirement for biofilm formation.

The specific mechanisms involved in social interaction and the molecular pathways on how microorganisms interact are referred to as cell-to-cell signalling, and in particular 'quorum sensing - QS'. QS is a mechanism in which microbial cells communicate, by releasing small signal molecules that diffuse freely across the cell membrane [223]. The QS system is based on cell density and requires a sufficient microbial population to produce an abundant concentration or 'quorum'. When attained, a quorum of signalling molecules triggers differential gene expression and behavioural traits that only occur whilst growing within a social community [218,224].

The increased synthesis of signalling molecules creates a positive feedback loop hence QS molecules have been termed autoinducers. Autoinducers are generally divided into three classes; the lux-type system in Gram-negative bacteria that utilise acyl-homoserine lactones (AHL) for species specific communication [225], autoinducing peptides (AIP) used by Gram-positive bacteria [226], and the luxS encoded autoinducer 2 (AI-2) enabling cross species communication between both Gram-negative and Gram-positive bacteria [227].

Gram-negative bacteria produce a unique AHL or a unique combination of AHLs (if it possesses more than one LuxI-type protein). As a result, only members of the same species recognize and respond to it. *P. aeruginosa* is the most widely reported AHL producing bacteria, with several of its virulence traits controlled by AHL, including rhamnolipid up-regulation.

Additionally, several other aspects of biofilm life have been linked with the AHL-QS system of *P. aeruginosa* including biofilm diversity, architecture, resistance and dispersal [228-230].

Gram-positive bacteria also use intercellular communication through the AIP mechanism. However, it is vastly more complex than its Gram-negative counterparts and requires greater depletion of energy resources [231]. The most widely reported of these systems is *S. aureus* and its encoder, accessory gene regulator (*agr*). This QS molecule is responsible for the production of a wide array of virulence factors [232,233], and has been cited for its role in biofilm maturation [234].

The role however of QS systems in biofilm development has been predominantly examined under planktonic cultures [235-238]. Data has indicated that QS modulates numerous microbial pathways, including the regulation of virulence factors, adhesion molecules [239] and swarming motility [235]. Davies *et al* (1998) [229] were amongst the first to hypothesise that cell-cell signalling could be involved in *P. aeruginosa* biofilm maturation. Davis *et al* (1998) identified that a *P. aeruginosa* mutant strain lacking QS production had a significantly thinner EPS (20%) that was more vulnerable to biofilm disruption through the application of a detergent.

Yarwood *et al* (2004) [234] studied the contribution of *agr* expression to biofilm development. Under certain conditions *agr* expression enhanced biofilm formation, conversely in other conditions *agr* expression had no effect on biofilm formation or seemed to impair biofilm growth. Tan *et al* (2015) identified that the absence of the *agr* function in methicillin sensitive *S. aureus* led to biofilms that were greater in density and more resistant to antibiotics [240]. Vuong *et al* (2004) [241] studied *S. epidermidis* mutant strains defunct of *agr* production, and found the absence of *agr* promoted biofilm development of in-dwelling medical devices. This data

suggests the mechanisms involved in QS and biofilm formation in Gram-positive bacteria differ in complexity, operating through numerous pathways. The use of QS inhibitors as part of anti-biofilm strategies for Gram-positive microorganisms may potentially promote biofilm formation [242].

The above QS systems refer predominantly to species-specific communication. The auto-inducer peptide (AI-2) has been described as a universal language allowing intra-species communication [227,243]. MacNab *et al* (2003) [244] highlighted the potential of intra-species communication through their *in vitro* model on polystyrene surfaces. Two mutant strains of *P. gingivalis* and *S. gordonii* missing the Lux-S gene required for AI-2 synthesis were compared to wild strains with intact Lux-S synthesis. In the absence of AI-2 mixed biofilm formation was not possible, unlike wild type strain microorganism that formed mixed species biofilm.

Despite the plethora of studies over the last three decades that have pursued the involvement of cell-to-cell signalling and its role in bacterial infections or biofilm formation, there has been little in the way of translational development of medicines, medical devices or other quorum sensing therapeutics that have made their way into commercial use for human health and disease. In fact, despite of the focus on QS, little is still known about its activity in human infections [245].

2.4.3. BIOFILM DISPERSAL: TO BOLDLY GO WHERE NO MICROORGANISM HAS GONE BEFORE

Within the biofilm life-cycle there is a point when over-population, crowding, or a lack of nutrients occurs [246]. There will also be microbes who transit to and from a biofilm in an attempt

to explore and colonize new niches [247]. The process of biofilm dispersion encapsulates the reversion from a sessile phenotype to a planktonic cell.

Biofilm dispersal can occur through passive processes such as erosion, a physical sloughing event (i.e. tooth brushing) or fluid shear [117]. Alternatively, biofilm dispersal may arise due to active mechanistic events triggered by the microbial biofilm themselves in response to environmental changes. These may include nutrient starvation, toxic by-products, pharmaceutical/medical device related treatments such as antimicrobial stress or unfavourable oxygen levels [121].

Boyd and Chakrabarty (1994) [248] investigated the role of alginate lyase in cell dispersion in *P. aeruginosa* biofilms. The production of alginate lyase in a mutant strain resulted in the disruption of EPS and increased microbial dispersal. Allison *et al* (1998) [228] also identified the production of two-exopolysaccharide lyase enzymes that degrade P. fluorescens biofilm and thus promotes microbial.microbial dispersion. In *S. epidermidis*, Coulon *et al* (2012) [247] identified a link between a reduction in nutrient availability and a reduced production of polysaccharide intercellular adhesion (PIA). PIA is an important component in cellular aggregation, and under nutrient starvation conditions, Coulon *et al* (2012) found that significant reductions in PIA were associated with rapid biofilm dispersal.

2.4.4. BIOFILM TOLERANCE

Antimicrobial therapies based on the routine microbiology laboratories reporting of the bacterial species in question's susceptibility to a disc diffusion test [249], target rapidly multiplying planktonic microorganisms with high efficacy. Unfortunately, when these

therapies are employed against biofilm phenotype microorganisms that differ markedly in both their physiology[250] and activity [251], they typically fail to eradicate the problem (chronic infection). Indeed, a plethora of *in vitro* biofilm models have elucidated that bacterial biofilms can withstand antimicrobial concentrations 100 to 1000 times higher than that of planktonic counterparts [145,172,252-254]. Both resistance [255,256] and tolerance [257-259] of biofilms have been reported synonymously in defining their ability to withstand much higher concentrations of antimicrobials (both topical, oral and intravenous), antiseptics and disinfectants, but they infer two very different mechanisms.

Individual bacteria can promote resistance through mobile genetic elements such as plasmids or transposons allowing horizontal gene transfer [260], or by target mutations, modifying enzymes or efflux pumps [261]. These familiar mechanisms by which individual planktonic microorganisms can resist the increased concentrations of antimicrobials do not seem to explain the enhanced protection afforded to bacteria in biofilm phenotype. Instead, an enhanced tolerance and ability of biofilms to survive concentrations 100 to 1000 greater then planktonic microorganisms have been directed towards mechanisms induced by their phenotypic state [262]. *In vitro* data has identified that when biofilm microorganisms revert back to a planktonic state, their susceptibility to antimicrobials also increases [263,264].

Despite the wealth of research undertaken to identify biofilm resistance and or tolerance to antimicrobials no single causative mechanism has been identified. Instead it has been suggested that a likely combination of factors contributes to biofilm tolerance [145,258,265]. The protective mechanisms of biofilm microorganisms to many forms of chemical and biological attack have yielded several areas of interest including but not limited to; slow or incomplete permeation of antimicrobials through extracellular polymeric substance (EPS), altered microenvironment and

niches within biofilms promoting slow growth rates and adaptive stress response, efflux pumps, and the role of “persisters”.

2.4.4.1. SLOW OR INCOMPLETE PERMEATION OF ANTIMICROBIALS THROUGH BIOFILMS

One focus on the ability for biofilms to withstand significantly greater concentrations of antimicrobials has singled out the protective force field of the EPS. The process of permeation that acts synergistically with enzymatic deactivation and the polyionic nature of EPS. The available literature suggests that the type of antibiotic precipitates the ability to penetrate EPS, and it simply seems that some antibiotics have a greater ability to penetrate biofilms and act against their targets, in comparison to some antibiotics that encounter difficulties.

In support of this, some researchers have explored the surface charge omitted by EPS as a mechanism that may lead to binding and/or deactivation of an antibiotic [172]. Most studies have centred on *P. aeruginosa*, and its alginate polymer that produces a negative charge that can bind and sequester positively charged antibiotics such as gentamicin [266]. Ciprofloxacin however, being uncharged, maybe less affected by alginate [267]. The ability of the quinolones (ciprofloxacin, ofloxacin), lipopeptides (daptomycin) and polypeptides (colistin) to rapidly penetrate biofilm and kill microbial cells at therapeutic dose levels, demonstrates the ability of some antibiotics to be effective against medically relevant biofilms [172,253,257,268].

Anderl *et al* (2000) [267] tested the penetration of ampicillin against a β-lactamase-positive *K. pneumonaie* wild type strain *in vitro*, revealing ampicillin deactivated quicker than it could diffuse through the EPS. Similarly, this has been identified in aminoglycoside antibiotics tested on β-lactamase-positive *P. aeruginosa* biofilms *in vitro* [269]. Additional data provided from

Singh *et al* (2010) [270] on *S. aureus* and *S. epidermidis* biofilms, have further identified reduced penetration of oxacillin, cefotaxime (β-lactams) and vancomycin (a glycopeptide) through *in vitro* biofilm models. Taken collectively, it seems negatively charged polymers of both *K. pneumonaie and P. aeruginosa* are effective in protecting microbial cells from positively charged agents through limiting their diffusion via binding [271].

2.4.4.2. ALTERED MICROENVIRONMENT AND NICHES WITHIN BIOFILMS

Advancements in technologies and techniques applicable to the study of *in vitro* biofilms in situ have allowed researchers to explore the interior of biofilms identifying niche areas of oxygen and nutrient depletion and altered pH levels. Microelectrode analysis of biofilm structures have identified that oxygen is depleted at the substratum layers of biofilms and in the centre of microcolonies [129,172,272]. Utilising methods such as microinjection of fluorescent dyes and quantitative analysis using confocal laser microscopy, de Beer *et al* (1994) explored local diffusion gradients developed in biofilms, identifying anoxic and acidic zones in the interior of biofilms [177].

These varied microenvironments can contribute to both nutrient limitations that promote limited stationary-phase physiology [273] and or effects to antimicrobial action. Antimicrobials are predominantly effective against rapidly growing microbial cells with some antibiotics such as penicillin and ampicillin requiring an absolute condition of microbial growth [258]. Walters *et al* (2003) [172] exemplified this when they tested the effects of tobramycin and ciprofloxacin against *P. aeruginosa* biofilms *in vitro*. Both antibiotics penetrated the biofilm but failed to effectively kill the bacteria, with antibiotic-affected cells being located exclusively near the air interface where most metabolically active cells are located. Altered metabolism and a reduced drug efficacy promoted by environmental niches and stresses therefore, undoubtedly contribute

to biofilm tolerance. Newer generation antibiotics such as daptomyocin and colistin can kill non-dividing biofilm cells as shown *in vitro* [253,274].

2.4.4.3. EFFLUX PUMPS

Efflux pumps expressed by planktonic microorganisms remove toxic substances such as antimicrobials, and their actions and roles in Gram-negative bacteria multi drug resistance are well documented [275] . Presently, little is known about the phenotypic changes that occur during the transition from the planktonic to the biofilm mode of growth but the role of efflux pumps in biofilm tolerance to antimicrobials has emerged as an area of interest. Whilst limited, current evidence indicates that efflux pumps are highly active in bacterial biofilms, making them attractive targets for potential anti-biofilm measures [276,277].

2.4.4.4. PERSISTER CELLS IN MICROBIAL BIOFILMS. AN ANSWER TO THE RIDDLE OF BIOFILM TOLERANCE?

Biofilm show enhanced tolerance to many forms of antimicrobials, antiseptics and disinfectants, as outlined by their ability to withstand 100 – 1000 times the minimum lethal dose. This would suggest that biofilm cells harbour the capability to switch on multidrug resistance mechanisms that they only express during biofilm growth. If this were true, then mutants expressing the same resistance would have been seen in rapidly multiplying planktonic cultures. This has not been the case, and has therefore led to the riddle of how a biofilm can be resistant to killing by many antimicrobials in the absence of specific resistance mechanisms [278].

An answer to this riddle has been proposed in the form of "persister" cells. Brooun *et al* (2000) [257] discussed the role of persister cells when they explored the dose-response of *P. aeruginosa* biofilms *in vitro* to ofloxacin and ciprofloxacin. Following exposure, a 3-log10 to 4-log10 reduction of bulk biofilm cells was achievable through therapeutic levels. However, following this initial log reduction, further increasing concentrations of antimicrobials were ineffective at eradicating surviving microbial cells.

From this simple observation of seemingly "invulnerable" cells, Lewis *et al* (2001) presented a rationale for persisters being responsible for biofilm tolerance to antimicrobials [257]. There are now numerous review articles that have outlined a rationale to further support the role of persisters in biofilm tolerance [145,258,265,278], and a few commonalities from across the literature is discussed below.

Initially identified in rapidly growing planktonic populations [279], and later in microbial biofilms [257,280], persister cells seem to exist as phenotypic variants of normal cells within a population and not as mutants. In fact, experiments have shown that perister populations when re-inoculated, produce a bulk of sensitive cells in addition to new tolerant cells [279-281]. While persisters are not unique to biofilms, the mere fact that biofilms are significantly more tolerant then planktonic microorganisms suggest that biofilms may produce more persisters [281-283]. Evidence to support this hypothesis is presented by data showing that persister formation is growth dependent and inversely related to metabolic activity [259]. Given that key elements to biofilm life are slow growth and reduced metabolic profiles, it seems plausible that biofilms promote persister formation.

One of the conundrums of biofilm resistance has been the inability to explain how biofilms are tolerant to treatments but lack specific multidrug resistance mechanisms. If persisters do not confer conventional multi drug resistance afforded to planktonic microorganisms, how do they potentially resist treatment? One explanation has been that persister cells decrease their metabolic profiles and enter a dormant state, thus reducing functional targets for the action of cidal antimicrobials [284]. In this respect, persister cells interfere with the lethal action of cidal antimicrobials by shutting down their target functions such as peptidoglycan synthesis [281], ribosomal synthesis and DNA replication [278]. Evidence supporting this has emerged from studies of *E. coli* where researchers have identified the toxin/antitoxin module *hip A* gene could shut down cellular functions in persister cells [281].

The role of peristers in biofilm tolerance to antimicrobials are still poorly defined. Their contribution to the recalcitrance of biofilms to treatment however, may explain in part a common clinical symptom noted in chronic biofilm-associated infections, relapse of infection. The use of antimicrobials against microorganisms will reduce the bulk of cells in the population or eradicate them completely. In acute infections caused by planktonic microorganisms, any remaining persisters are likely to be exposed to the host immune system, which “mop up” the remaining persisters.

Herein lies a problem for the host challenged by biofilm and compounded in those patients who are immunocompromised. The use of antimicrobials may desiccate the bulk of biofilm and planktonic cells. Clinically, this may be represented by improvements in visual symptoms of infection. Following the discontinuation of antimicrobial therapy, the remaining persisters sheltered from the host immune response through the EPS reform the biofilm that acts as a

nidus for shedding planktonic microorganisms, and an acute infective flair may ensue [156,258,278,285].

2.5. ANTIMICROBIALS AND OTHER AGENTS USED IN WOUNDCARE AS ANTI-BIOFILM STRATEGIES

Topical antimicrobials or antiseptic wound dressings are commonly used by clinicians to target biofilms associated with chronic non-healing wounds. The use of topical antimicrobial or antiseptic solutions to decontaminate wounds has been around for millennia, but the use of such agents with specific context to managing wound biofilm has increased significantly in recent years. This has largely been, in part, due to the marketing campaigns of many companies who have claimed their topical solution has anti-biofilm properties. The action and effectiveness of agents as bactericidal or bacteriostatic agents in targeting biofilm-phenotype bacteria are based solely on *in vitro* and animal models. To the best of our knowledge, this thesis is the first to perform an *in vivo* human wound study that explores the effects of antimicrobial agents against wound biofilm, and explore effectiveness using 16S rDNA sequencing and microscopy methods.

When critiquing the evidence to substantiate anti-biofilm claims, there are large gaps in the available literature. This is discussed in in section 2.6 and Chapter 7. There is limited *in vitro* and *in vivo* evidence (using standardized biofilm models) of the performance of antimicrobial solutions marketed and used for the sole purpose of wound-care agents. More traditional antiseptics such as Chlorhexidine and Povidone Iodine, though, have been tested against a multitude of human and animal wound related biofilms [286-288] and oral biofilms [289-291]. This data has reported that both antiseptics are effective *in vitro* and in animal models and will only be discussed further where directly applicable to wound-care related usage.

2.5.1 TOPICAL ANTIMICROBIAL DRESSINGS / AGENTS

Ionic silver has been widely studied as a topical antimicrobial treatment used by clinicians to cater for a wide array of wound aetiologies complicated by high levels of planktonic microorganisms. The emergence of biofilms as a concept in delayed wound healing and as contributors to infection has seen a concomitant application to studying the effects of ionic silver on biofilm phenotypes. Early reports for the use of ionic silver in *S. epidermidis* biofilms suggested that low concentrations of silver ions were unable to eradicate mature biofilms (0.05 ppm of silver) [292].

Bjarnsholt *et al* (2007) [293] tested the performance of multiple ionic silver dressings against four-day-old *P. aeruginosa* biofilms *in vitro*. Biofilms were exposed to 10 ppm of silver sulfadiazine and complete eradication at this concentration was noted. Following this, exposure of biofilm to three commonly used ionic silver wound dressings (varying degrees of ionic silver concentration - a. 0.033 ppm, b. 2.2 ppm, c. 0.93 ppm) found no effect on biofilm disruption. Non-healing chronic wounds may often create excessive exudate (both primarily linked to biofilms and or due to other systemic factors) that contain chloride ions. These are known to inactivate ionic silver through forming insoluble compounds of silver chloride [294]. For this reason, Bjarnsholt *et al* (2007) concluded that both higher concentrations of ionic silver and the mode of delivery were important factors in wound dressings likely to affect biofilm.

Percival *et al* (2008) [295] used a sustained release ionic silver dressing (AQUACEL® Ag, Convatec inc, US) containing 1 ppm to assess the *in vitro* effects against biofilms of *P. aeruginosa*, *S. aureus*, *E. cloacae* and a mixed species biofilm. A 24-hour biofilm model was subjected to the sustained release silver dressing and LIVE/DEAD staining was used to confirm

the presence of live or dead cells. Within 24 to 48 hours' post application to all biofilm models, a 90% and 100% kill rate of sessile bacteria were noted. A major limitation of this study was the absence of incorporating any nutritional or media flow in the biofilm model to mimic wound exudate. The absence of media would create a bias environment by restricting exposure of ionic silver to chloride binding allowing higher concentrations of ionic silver then those expected in a wound.

Hill *et al* (2010) [294] tested the effectiveness of various topical antimicrobial wound dressings *in vitro* on mature *S. aureus* and *P. aeruginosa* biofilms in a constant depth film fermenter. This methodology attempted to replicate the presence of exudate in chronic wounds by partially soaking the dressings in a controlled amount of protein rich media. This media was replaced daily for up to seven-days and the authors proposed this was a more accurate reflection of a real wound environment. In total, six silver dressings were tested on mixed species biofilms and the results indicated that exposure to the silver dressings over three and seven-days had no effects on biofilm disruption. Hill *et al* (2010) concluded that protein rich media likely reduced the bioavailability of ionic silver. In the same experiment, Hill *et al* (2010), tested Iodine based products (PVP-I, Acelity and Cadexomer Iodine®, Smith and Nephew). In contrast to the poor results noted against silver wound dressings, both Iodine based antimicrobials eradicated *S. aureus* and *P. aeruginosa* biofilms at three and seven-days exposure.

Kostenko *et al* (2010) [296] tested the performance of five different silver dressings over a seven-day period against bacterial biofilms of *P. aeruginosa*, MRSA and *E. coli*. Additionally, the silver dressings and bacterial biofilms were transferred into fresh media daily to represent a continual exposure to fresh wound exudate. The results identified all the silver dressings were unable to decrease the number of sessile cells within a 24-hour period ($p < .05$). Nanocrystalline

silver yielded the greatest log reductions (4 log10 - 5 log10) and maintained the reduction over the seven-day period. The remaining silver dressings also reduced sessile cell numbers over the first 24-hours (2 log10 - 3.5 log10), but after 24 hours their actions rapidly deteriorated and biofilm re-growth was observed.

Phillips *et al* (2015) [297] used an *ex vivo* porcine skin explant model on mature three-day *P. aeruginosa* PA01 biofilms, to test multiple topical antimicrobial agents commonly used in woundcare; Cadexomer Iodine, PVP-I, silver, PHMB, honey and alginates. Dressings were exposed for 24 and 72-hour continuous exposure and the bacterial cell viability was determined using cfu. Only Cadexomer Iodine could fully eradicate *P. aeruginosa* PA01 biofilms at both 24 and 72-hours exposure. Nanocrystalline silver dressings could reduce cfu by >2 log10 at 24 and 72-hours, but all other dressings were unable to achieve a log reduction >1 log10.

Fitzgerald *et al* (2017) [298] evaluated the performance of Cadexomer Iodine, Povidone Iodine, ionic silver and PHMB against 48- hour *S. aureus* and *P. aeruginosa* biofilms in a multi experimental design incorporating; an *in vitro* colony biofilm model, colony drip-flow biofilm model, *MRSA* colony wound biofilm model and a mouse wound biofilm model. Bacterial cell viability was determined by cfu. 24-hours exposure to Cadexomer Iodine resulted in full eradication of *S. aureus* and *P. aeruginosa* biofilms in the colony biofilm model. In comparison, other dressings were only able to achieve <2 log10 reduction.

In the colony drip-flow biofilm model, exposure of Cadexomer Iodine for 24-hours resulted in a >5 log10 reduction against both *S. aureus* and *P. aeruginosa* biofilms, whilst the other dressings were not able to achieve >0.7 log10 reductions. Similarly, in the *MRSA* colony wound biofilm model Cadexomer Iodine achieved >7 log10 reductions in comparison to other

dressings who could not achieve >1 log10 reductions. Lastly, in a mouse MRSA wound biofilm model the application of Cadexomer Iodine for a 48-hour exposure resulted in >4 log10 reduction in comparison to other treatments (0.7 - 1.6 log10 reduction).

Hoekstra *et al* (2017) [299] evaluated the performance of 10% PVP-I ointment (at concentrations 3.3%, 10% and 33% and 100%) and silver nanocrystalline in a CDC biofilm reactor against 48-hour mixed biofilms of *P. aeruginosa* and mixed biofilms of *MRSA* and *C albicans.* Following exposure of the agents to *P. aeruginosa* biofilms for 24-hours, bacterial cell viability (cfu/mL) identified that 10% PVP-I (all concentrations) eradicated biofilms (>5 log10 reduction), whereas silver nanocrystalline reduced biofilms by 2 log10. Against mixed species biofilms, 24-hour exposures both 10% PVP-I (100% concentration) and silver nanocrystalline resulted in full eradication of biofilms (>5 log10 reduction). 10% PVP-I tested at reduced concentrations (3.3% and 33%) identified variable results against mixed species biofilms only achieving a <2 log10 reduction. One of the major strengths of the study were to test PVP-I at differing concentrations to account for the presence of wound fluid. This is because the effects of wound fluid cause dilution or they inactivate agents.

Using a colony drip-flow biofilm reactor, Bourdillion *et al* (2017) [300] evaluated the performance of ionic silver dressings, nanocrystalline silver dressings and Cadexomer Iodine, against 24 and 72-hour *P. aeruginosa* biofilms. Against 24-hour biofilms PROMOGRAN PRISMA™ (oxidised regenerative cellulose with 0.02 ppm of silver) and ionic silver reduced *P. aeruginosa* biofilms by 0.2 log10, but Cadexomer Iodine was not tested. After 72-hour PROMOGRAN PRISMA™ performed better than all the other dressings by reducing 24-hour *P. aeruginosa* biofilms by 1.49 log10 (p = 0.01), whilst Cadexomer Iodine were only able to reduce *P. aeruginosa* biofilms by 0.8 log10. These results are surprising given the previous

evidence for the performance of Cadexomer Iodine against mature biofilms both *in vitro* and in animal models. Furthermore, PROMOGRAN PRISMA™ contains only 0.02 ppm of silver, the lowest concentration of silver bioavailability in any wound product, yet it could achieve log reductions greater than wound dressings containing 100 – 1000 times the silver concentrations and perform better then Cadexomer Iodine.

Given the results conflict with many other published data, the authors fail to discuss the relevance of their findings in the relation to past data. No attempts were made to discuss or explain the poor performance of Cadexomer Iodine and why this contrasts with many other *in vitro* and animal data. The authors also fail to explain the results of log reductions in an appropriate context to what is an effective log reduction for an *in vitro* test yet make the claim that PROMOGRAN PRISMA™ was the only dressing to significantly reduce biofilm populations.

However, when interpreting these results, the starting amount of 24-hour *P. aeruginosa* biofilms pre-treatment were 6.73 log10 and post-treatment following PROMOGRAN PRISMA™ was 6.47 log10. This is only a 0.2 log10 reduction which indicates a poor effect. The performance of PROMOGRAN PRISMA™ against 72-hour *P. aeruginosa* biofilms identified a 1.49 log10 reduction from a starting amount of 9.30 log10, therefore leaving 7.5 log10 of biofilm bacteria. In this context, the performance of PROMOGRAN PRISMA™ remains poor.

2.5.2 TOPICAL ANTIMICROBIAL / ANTISEPTIC SOLUTIONS USED IN WOUNDCARE

Of the available *in vitro* data on topical antimicrobial solutions, Sauer *et al* (2009) [301] present a study testing the performance of super-oxidised solution (Microdacyn™) using a continuous

flow tube reactor on mature six-day *P. aeruginosa* PA01. Super-oxidised solution of varying concentrations of the active ingredient hypochlorous acid/sodium hypochlorite (80 ppm, 125 ppm, 200ppm), were tested under flow against *P. aeruginosa* PA01 biofilms. Outcome measures for bacterial cell viability were cfu and LIVE/DEAD stain reported at 5-minute intervals for up to 60 minutes' total exposure. The highest concentration of SOS (200 ppm) used for a 60-minute exposure resulted in > 3 log10 reduction in cfu and a 12.7-fold reduction in biomass.

The above findings on the performance of SOS would seem to suggest its effectiveness *in vitro* against *P. aeruginosa* PA01 biofilms, and the evidence from this paper is used for promotion by a medical device company manufacturing SOS. The problem with the above results, however, is that they lack clinical relevance and applicability for use in wounds. Firstly, the tube flow reactor is an experimental model designed to look at either disinfection of hospital-based equipment and or catheter-based biofilm models. Secondly, the outcomes reported for 60 minutes do not reflect a clinically relevant time for the use in wound-care, of which a typical exposure time would last for a maximum of 15 minutes (as promoted for intended usage by medical device companies).

When the data is re-analysed at these clinically relevant exposure times, a different story begins to appear. The performance of SOS (200 ppm) at 10-minute and 15-minute exposure is 0.65 log10 and 1 log10 respectively, which is significantly less than the >3 log10 observed at 60-minute exposure time. Thirdly, the concentration which exhibited the greatest effects of SOS was 200ppm, and this represents the industrial strength used for disinfection purposes. The concentration of SOS used in wound solutions is 65-80 ppm, a concentration for which no data are available.

Davis *et al* (2017) [302] tested the effectiveness of three topical antimicrobial wound solutions (Polyhexamethylene biguanide, PHMB - Prontosan®, 60 ppm hypochlorous acid/sodium hypochlorite – Microdaycn®, Octenilin dihydrochloride - Octenilin®) against immature 24-hour *MRSA* (ATCC 33593) biofilms in a porcine wound model. Each porcine wound was irrigated twice daily for six days' total with the antimicrobial solutions, and bacterial cell viability was determined by cfu/g on days three and six from baseline initial biofilms counts of 7.42 log10 cfu/g.

Treatment with PHMB reduced biofilms by 1.67 log10 at day three and 3.08 log10 reduction at day six. Treatment with Octenilin® reduced biofilms by 0.76 log10 at day three and 2.56 log10 at day six. Treatment with hypochlorous acid/sodium hypochlorite increased the cfu/g after three days of treatment by 0.24 log10 (pre-treatment = 7.42 log10 cfu/g vs post-treatment 3 days = 7.66 log10 cfu/g), but after six days of treatment a reduction of 0.67 log10 was noted. The authors conclude that PHMB performed better and was superior to other antimicrobial wound solutions for use against wound biofilm. While this statement attempts to paint a positive light for specific antimicrobial solutions, the performance in reducing biofilms is questionable. None of the solutions could achieve >3 log10 reduction against immature biofilms of 24 hours following treatments of up to six days. However, the results are not surprising given the extremely short exposure times of the solutions which were used as irrigations (exposure of seconds) as this exposure time is likely inadequate [303].

2.5.3 OTHER MEDICAL DEVICES USED FOR WOUND BIOFILM TREATMENT

Negative pressure wound therapy (NPWT) is utilised by clinicians to improve wound healing in several clinical pathologies. In the diabetic foot, it is commonly employed post-operatively

to augment debridement or surgical procedures by enhancing angiogenesis and granulation tissue formation [304], but may also be employed for use in chronic wounds to promote wound healing [305]. As its title suggests, its primary mode of action is providing a topical negative pressure to the wound interface via a special dressing system consisting of a permeable foam (some foams dressings can have impregnated silver) and tube system which encloses and seals the wound. Ngo *et al* (2012) [306] hypothesised that the action of NPWT would likely lead to an alteration to biofilm architecture.

P. aeruginosa (ATCC 25619) biofilms were grown under shear in a CDC biofilm reactor on polycarbonate discs for 24-hours and then transported to an *in vitro* wound model. The wound model incorporated bacteriological agar to mimic a wound surface allowing the biofilm-containing polycarbonate discs to embed. A nutrient in-flow system provided continuous flow of 1% TSB and the polycarbonate discs were sealed with 1) control no NPWT, 2) NPWT with foam and 3) NPWT with silver impregnated foam. Outcomes measures were assessed at day one application and day seven using cfu and LIVE/DEAD stain with CLSM to determine bacterial cell viability, average biofilm thickness and average and maximal diffusion distances.

Following a seven-day application of topical negative pressure, there was no significant reduction in cfu between control and those receiving topical negative pressure with foam (control = 1.18×10^7 vs NPWT and foam = 1.19×10^7). NPWT with foam, however, altered biofilm characteristics, decreasing the average thickness of biofilms, and the average and maximal diffusion distances ($p = 0.001$). When biofilm coupons were challenged with NPWT and silver foam, there was a significant reduction in viable bacteria in one day by 3 log10 and by day seven no viable bacteria were present ($p = .002$). The authors suggest the alteration to

biofilm architecture by topical negative pressure provides an enhanced synergy with topical silver to provide a greater effect against *P. aeruginosa* biofilms.

Phillips *et al* (2013) [307] used an *ex vivo* porcine skin explant model to test the performance of antimicrobial solutions under negative pressure wound therapy with instillation (NPWTi) against mature *P. aeruginosa* PA01 biofilms. NPWTi was applied for six cycles over 24-hours with each 4-hour cycle consisting of 30 seconds of instillation followed by ten minutes of dwell and four hours of continuous negative pressure at 125 mm Hg. The solutions tested were 1% PVP-I, 10% PVP-I, PHMB and chlorhexidine gluconate, and these were compared to untreated coupons and coupons receiving NPWT alone. Control coupons bacterial loads were 7 log10 with NPWT alone reducing bacterial load <1 log10. Instillation with 10% PVP-I resulted in the largest reductions to bacterial loads (5 log10), followed by PHMB (4 log10), chlorhexidine gluconate (3 log10) and 1% PVP-I (2 log10). This data provides support to the concept raised by Ngo *et al* (2012), suggesting that the effects of NPWT to biofilm architecture *in vitro* may also occur when employed against tissue (in the form of porcine skin). To date, no trials *in vivo* on human subjects have been undertaken to evaluate the potential synergy gained from NPWT.

2.5.4 WOUND DEBRIDEMENT

Wound debridement using a scalpel blade or curettage has long been regarded as the gold standard practice for managing chronic DFUs without ischemia by removal of devitalised tissue and thus bacteria [308]. A single report on sharp debridement has identified positive effects on mature biofilm [309]. Findings from this clinical case series on three chronic VLUs complicated by *P. aeruginosa* biofilms, identified sharp debridement resulted in a 4 log10 reduction in biofilm bacteria, but biofilms reconstituted within 24 hours. Interestingly at 24

hours' post debridement, biofilm susceptibility to gentamicin increased. A nine-fold reduction in antibiotic concentration was required to induce biofilm disruption in comparison to a control biofilm. Correlating this finding clinically, explains the potential mechanism of sharp debridement to physically disrupt and remove parts of a biofilm, forcing it to reconstitute itself with younger cells that are more susceptible to antimicrobial attack. The authors proclaim that debridement opens a therapeutic window when antimicrobial treatments may be more successful.

In some patients in whom sharp debridement is not possible (i.e. wound ischemia, wound pain), treatments are often augmented with agents that are chemical or biological and that can aid in providing autolytic debridement. Yang *et al* (2017) [310] explored the effectiveness of a surfactant based poloxamer gel (autolytic debriding agent) in reducing mature *P. aeruginosa* PA01 biofilms in an *ex vivo* porcine skin explant model. The gel was placed on wounds daily and gently wiped and re-applied to mimic what would occur at a dressing change. Following three days of treatment observed bacterial cell viability (cfu counts) identified a total reduction/eradication of *P. aeruginosa* PA01 biofilms ($p = .02$).

Wilkinson *et al* (2015) [311] tested the effectiveness of two mechanical polymer fiber debriding devices (Debrimitt™ and Debrisoft™) against mature *P. aeruginosa* and *S. aureus* biofilms in a porcine skin explant model. Mechanical debridement using Polymer fiber pads were compared against sterile gauze using a mechanical brush simulator to control; levels of force, direction of debridement, duration of debridement. Biofilm removal was determined using a surface viability assay, bacterial counts (CFU), histological assessment, and scanning electron microscopy (SEM).

The results identified that the polymer fibre pads significantly reduced viable *P. aeruginosa* biofilm surface coverage compared to control biofilms that underwent no debridement (Debrimitt™ $p = .003$ and Debrisoft™ $p = .001$). No significant difference was found between the gauze dressing and control *P. aeruginosa* biofilms. Post-debridement on *S. aureus* noted a reduction in the biofilm surface coverage but this was not statistically significant.

Bacterial viability counts (CFU) against *P. aeruginosa* biofilms identified that both Debrimitt™ and Debrisoft™ treatment appeared effective, resulting in a 6 log10 reduction ($p < 0.001$), and 5 log10 reduction ($p < .001$) respectively. *S. aureus* viability was also significantly reduced (7 log10 reduction, $p < .001$) following debridement with both Polymer fibre pads. By contrast, treatment with a gauze dressing led to a 5 log10 reduction in *S. aureus* ($p < .001$).

Both SEM and confocal microscopy further identified that treatment with Polymer fiber pads significantly altered biofilm architecture of both *P. aeruginosa* and *S. aureus* biofilms, but sterile gauze did not. In particular, a substantial proportion of the bacteria and EPS were removed using Polymer fibre pads. Using a porcine skin explant model, the data suggest that Polymer fibre pads help remove both EPS and wound bacteria, thus reduce viable biofilms of *S. aureus* and *P. aeruginosa* from porcine tissue. The positive results noted here warrant further exploration from *in vivo* human trial. This would significantly enhance the clinical care of many patients with chronic non-healing wounds, because many woundcare clinicians are unable to perform sharp debridement (either through lack of competency or due to professional constraints) [11].

CHAPTER 3

3.1 16S RDNA NEXT GENERATION SEQUENCING OF TISSUES FROM INFECTED DIABETIC FOOT ULCERS

(APPENDIX 5 & 6) STUDY PROTOCOL AND ETHICS APPROVAL

(APPENDIX 7) **MALONE, M.,** JOHANI, K., JENSEN, S. O., GOSBELL, I. B., DICKSON, H. G., HU, H., VICKERY K. NEXT GENERATION DNA SEQUENCING OF TISSUES FROM INFECTED DIABETIC FOOT ULCERS. *EBIOMEDICINE* 2017. HTTP://DX.DOI.ORG/10.1016/J.EBIOM.2017.06.026

Candidate contribution:

- ✓ Worked as primary author for publication
- ✓ Designed the study protocol and methodology
- ✓ Performed all aspects of clinical study, including screening, recruitment, data collection, tissue biopsy sampling and storage.
- ✓ Used genomic software to quality control and analyse DNA sequences from raw files.
- ✓ Used bio statistical approaches to analyse genomic data
- ✓ Used statistical software and knowledge of statistical approaches to analyse / correlate clinical data
- ✓ Wrote the manuscript in full using information from thesis chapters 1 and 2
- ✓ Reviewed and amended all required changes from co-author contributions
- ✓ Submitted the manuscript as primary author

Abstract

We used 16S rDNA next generation sequencing to profile the microbiota of infected Diabetic Foot Ulcers (DFUs). The microbiome was correlated to clinical parameters and treatment outcomes to determine if directed antimicrobial therapy based on conventional microbiological cultures are relevant based on genomic analysis. Patients ≥18 years presenting with a new Diabetic Foot Infection (DFI) who had not received topical or oral antimicrobials in the two weeks prior to presentation, were eligible for enrolment. Tissue punch biopsies were obtained from infected DFUs for analysis.

Demographics, clinical and laboratory data were collected and correlated against microbiome data. Thirty-nine patients with infected DFUs were recruited over twelve-months. Shorter duration DFUs (<six weeks) all had one dominant bacterial species (n = 5 of 5, 100%, $p <$ ·001), *S. aureus* in three cases and *S. agalactiae* in two. Longer duration DFUs (≥six weeks) were diversely polymicrobial (p = ·01) with an average of 63 (range 19-125) bacterial species. Severe DFIs had complex microbiotas and were distinctly dissimilar to less severe infections (p = ·02), characterised by the presence of low frequency microorganisms.

Nineteen patients (49%) during the study period experienced antimicrobial treatment failure, but no overall differences existed in the microbiota of patients who failed therapy and those who experienced treatment success (p = .2). Our results confirm that short DFUs have a simpler microbiota consisting of pyogenic cocci but chronic DFUs have a highly polymicrobial microbiota. The duration of a DFU may be useful as a guide to directing antimicrobial therapy.

3.1.1. INTRODUCTION

In a person with diabetes a “triad” of factors that include peripheral neuropathy, peripheral vascular disease and trauma [2], place the foot at risk of developing a wound. Infections of the feet in people with diabetes are the primary pathway to lower extremity amputation [3]. Management of diabetic foot infection (DFI) is underpinned by the requirement to identify the pathogen/s of infection and thus direct antimicrobial therapy. Laboratory based methods that are culture-dependent are commonly utilised to identify microorganisms that are potential pathogens of infection, in addition to examining their density through qualitative and quantitative measures. This has shown acute ulcers are usually colonised by *S. aureus* and/or *S. agalactiae*, and chronic ulcers have a more diverse microbiota, with anaerobic organisms and *P. aeruginosa* becoming more important [25]. Culture-dependent techniques select for species that flourish under the typical conditions of the diagnostic microbiology laboratory, and this may not necessarily reflect the most abundant or clinically important microorganisms in DFIs especially anaerobes and species not detected under standard clinical microbiology laboratory protocols [312].

Molecular DNA based techniques that are culture-independent have identified the limitations of methods when examining the microbiota of wounds. Using amplification and sequence analysis of 16s rDNA, a highly-conserved gene present in all prokaryotes (bacteria) but not eukaryotes (humans), has revealed a vastly more complex array of bacterial communities in non-infected chronic wounds [90]. No data exists for acutely infected DFUs using this methodology.

We explored the microbiota of infected DFUs using 16S rDNA next generation sequencing. Data is presented on the microbial diversity, community structure, bacterial load and presence of likely pathogens from diabetic foot infections. Molecular findings are correlated against clinical factors and treatment outcomes.

3.1.2. MATERIALS AND METHODS

Patients, samples and ethics

Individuals presenting to a tertiary referral hospital (Liverpool Hospital High Risk Foot Service and Liverpool Hospital Emergency Department) with a newly infected diabetic foot ulcer occurring below the malleolus [2] were recruited consecutively over a twelve-month study period between January 2015 and December 2015. A 3mm (width) x 10mm (depth) tissue punch biopsy was obtained from the edge of each DFU after debriding and cleansing the wound with NaCl 0. 9%. Patients who had received any systemic or topical antimicrobial therapy two weeks prior to enrolment were excluded. Ethics approval for this study is noted in Appendix granted by the South West Sydney Local Health District Research and Ethics Committee (HREC/14/LPOOL/487, SSA/14/LPOOL/489). The study methodology was designed in guidance by STROME-ID and our molecular surveillance data are reported in keeping with this [313].

Patient demographic, laboratory and clinical data

Patient demographics, laboratory and clinical data were collected through patient charts and the electronic medical records for correlation against microbiota data. Clinical data and wound metrics of interest that were collected included; present or absent foot pulses, foot Doppler waveforms, toe brachial indices (TBI) and completion of the modified neuropathic disability

score [314]. DFU location, duration of DFU prior to presentation, size (length x width in mm), depth (mm) and tissue type (granulation, slough, necrosis). Laboratory data included; full blood count, inflammatory markers (White cell count [WCC], Erythrocyte sedimentation rate [ESR], C-reactive protein [CRP]), glycosylated haemoglobin (HbA1c) and estimated glomerular filtration rate (eGFR). All newly infected DFUs were diagnosed clinically, and their severity graded using the Infectious Disease Society of America Guidelines for DFI [2]. Acute infections were defined based on new presenting symptoms (classic signs of infection) being present and untreated of less than fourteen days' duration.

DFUs were classified based on their duration, with shorter duration DFUs (Acute) being less than six weeks and longer duration DFUs (Chronic) defined as those greater than six weeks. Treatment failure during the study period were defined as no resolution of infective symptoms over an appropriate treatment period (>28 days) despite directed anti-infective treatment [2], a requirement to replace oral antimicrobial therapy with parenteral delivery due to deterioration of infective symptoms, or the need for surgical intervention.

Tissue processing workflow

DNA Extraction

5 – 10 mg of human chronic DFU biopsy samples were defrosted on ice prior to DNA extraction. Genomic DNA was extracted using Mo Bio PowerBiofilm DNA isolation kit (Mo Bio, Carlsbad, CA, United States) following the manufacturer's instructions.

16S rDNA next generation sequencing to determine bacterial diversity

DNA sequencing was carried out by a commercial laboratory (Australian Centre for Ecogenomics, Brisbane, Australia) targeting the V3-V4 region of the 16S rDNA using

eubacterial universal primers 515F and 806R [315]. Preparation of the16S library was performed using the workflow outlined by the manufacturer (Illumina, San Diego, CA, United States).

In the 1st stage, PCR products were amplified according to the specified workflow with an alteration in polymerase used to substitute Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, Massachusetts, United States). Resulting PCR amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, United States). Purified DNA was indexed with unique 8bp barcodes using the Illumina Nextera XT 384 sample Index Kit A-D (Illumina, San Diego, CA, United States) in standard PCR conditions with Q5 Hot Start High-Fidelity 2X Master Mix. Indexed amplicons were pooled together in equimolar concentrations and sequenced on the Illumina MiSeq platform using paired end sequencing with V3 300bp chemistry.

Sequence analysis and quality control

Reads in FASTQ format were imported to CLC genomics workbench version 8.5.1 using the microbial genome finishing module (CLC bio, Qiagen Aarhus, Denmark), for sequence quality control and analysis. Workflows for sequence quality control and operational taxonomic units (OTU) clustering were based on previously reported wound microbiota analysis [10]. OTUs were defined as molecular proxies for describing organisms based on their phylogenetic relationships to other organisms and were reported at either the genera or species level identification where possible.

Sequence and quality control measures were performed using CLC genomic software. Reads were paired, merged and fixed trimmed at set averages of greater than 230 base pairs. OTUs were clustered, chimeric sequences removed and OTUs aligned using SILVA [316] at 97%

similarity to identify microorganisms at the genus level (species level where possible). OTUs were defined as molecular proxies for describing organisms based on their phylogenetic relationships to other organisms [10]. Where OTUs of interest such as *Staphylococci* which were only clustered at the genera level, each genomic sequence was manually reviewed (read length and >300 nucleotides) and utilised for analysis and further referenced for speciation using NCBI Mega BLASTtn [10]. This resolved a proportion of *Staphylococci* cases which speciation was possible, but overall species determination was limited. To classify microorganisms based on their residing origin at the genera level (i.e. skin, gut, environment, oral), microorganisms were manually referenced against Bergey's manual of systematic bacteriology volumes 1-5 [317-321].

Two sets of descriptive data were reported based on the relative abundance OTUs contributed to each individual wound (OTUs contributing 1% - 10% - these were considered major contributors, the second data set reports OTUs contributing ≥10% - these were considered dominant contributors) [10]. Next, OTUs were aligned using MUSCLE [10] to reconstruct a phylogenetic tree, and then subsampled allowing the estimation of the alpha and beta diversity. This included both community richness (Rarefaction) and community diversity (Shannon Weaver Index). Rarefaction curves allow the estimation of the number of unique microbial taxa within a sample and the Shannon Weaver Index is a measure of diversity that includes the number of unique microbial taxa and their relative evenness within each sample. Thus, a higher Shannon Weaver Index correlates to a greater diversity in a sample.

16s rDNA quantitative real-time PCR to determine microbial load

We utilised real-time quantitative PCR (qPCR) using the 16s rDNA eubacterial universal primers 341F 5'-CCTACGGGAGGCAGCAG-3' and 534R 5'-ATTACCGCGGCTGCTGG-

3’ to amplify a 194bp amplicon of 16s rDNA of all bacteria to determine the microbial load in DFUs as previously reported [322,323]. The total number of bacteria was expressed as per mg of tissue normalised to the average number of copies of the 18S rDNA gene in a mg of human tissue. The primer pair used in 18s rDNA real-time PCR was 18s rDNA_756F 5’-GGTGGTGCCCTTCCGTCA-3’ and 18s rDNA_877R 5’-CGATGCGGCGGCGTTATT-3’ to amplify a 122bp amplicon. 16S rDNA copy number per mg tissue were normalised to human 18s rDNA copy number per mg tissue.

Real-time PCR was carried out in 25 µl reaction mix containing 1X Brilliant II Sybr Green qPCR Master mix (Agilent Technologies, Santa Clara, US), 400nM forward and reverse primer and 100ng DNA template was analysed on the Mx3000P system (Agilent Technologies, Santa Clara, US) with the following cycling conditions: activation of Taq polymerase at 95^{o}C for 10 min, followed by 40 cycles of denaturation at 95^{o}C for 15 seconds, annealing at 56^{o}C for 30 sec and extension at 72^{o}C for 30 sec.

Each qPCR was run with standard samples of known concentrations (copies/µl). Ten-fold serial dilutions of the quantified 16s rDNA and ten-fold serial dilutions of 18s rDNA PCR amplicon solution was kept in aliquots at -20^{o}C and used as external standards of known concentration (copies/µl) in real-time PCR reaction. The standard samples were ranged 10^{2}–10^{6} copies/µl which used to construct a standard curve for each qPCR run. The calibration curve was created by plotting the threshold cycle (Ct) corresponding to each standard vs the value of their corresponding gene concentration (copies/µl). The copy number of 16s rDNA (copies/µl) was normalised against copy number of human 18s rDNA (copies/µl) in each wound sample.

Culture-dependent Bacteriological Enumeration and Identification

Culture-dependent analysis of wound punch biopsies was performed by a hospital pathology service (Sydney South West Pathology Service) using methods previously described.[163] Briefly, tissue samples were weighed and homogenized using a sterile tissue pulper in 3 ml of sterile saline. Serial dilutions of (1:10, 1:100 and 1:1000) of homogenized tissue was made, and two sets of plates were inoculated, one for the neat crushed tissue and one for the dilutions. Plates were streaked for isolation onto four quadrants of recommended agars and grown under appropriate atmospheres to isolate clinically relevant organisms (both aerobe and anaerobe) per standardized methods [324]. The number of microorganisms were quantified by colony forming units (cfu) and reported as either $\geq 10^6$ cfu/g of tissue or $<10^6$ cfu/g of tissue for each isolate.

Statistics

CLC genomics workbench version 8.5.1 in combination with the microbial genome-finishing module (CLC bio, Qiagen Aarhus, Denmark) were used to analyse DNA sequence data. Operational taxonomic units (OTU) clustering were based on previously reported wound microbiota analysis [10]. OTUs were defined as molecular proxies for describing organisms based on their phylogenetic relationships to other organisms. Associations between microbiota community structure and membership were compared using permutational multivariate analysis of variance (PERMANOVA) in combination with principal coordinate analysis PCoA Bray-Curtis dissimilarity matrix. Patient demographics, laboratory and clinical data were examined using Chi-square and Spearman correlation coefficients. Kappa coefficients were used to determine the level of agreement between culture-dependent approaches and DNA sequencing. Independent predictors of treatment failure were explored using general linear model (GLM). Mann Whitney U test for non-parametric data were undertaken when analysing

the subgroups of neuropathic or neuroischemic lesions. Analysis was performed using Statistical Package for Social Sciences (SPSS) Version 23, SPSS Inc., Chicago, Illinois, USA. For all comparisons and modelling, the level of significance was set at $p < .05$. Molecular data analysed through Bray-Curtis and PERMNOVA incorporated a Bonferroni correction. Data are given as mean, median and standard deviation (±).

3.1.3. RESULTS

39 patients (39 tissue specimens) with newly infected DFUs were recruited over the 12-month study period. Broad demographic, clinical and laboratory data are shown in Table 3.1.

Table 3.1 Patient demographics, clinical and laboratory data for 39 patients presenting with diabetic foot infection. Systemic antimicrobial use were based on post-sampling rtherapeutic regimens.

Characteristics	**n=patients (%) (±SD)**
Demographics	
Mean age	57.4 years (±11·5)
Male/Female	28 (71%)/11 (29%)
Type of Diabetes: Type 1/Type2	4 (10%)/35 (90%)
Duration of diabetes	12.8 years (±6·5)
Chronic Kidney Disease Stage 5	16 (27%)
Duration of ulcer prior to presentation	15.7 weeks (±13·7)
Co-morbidities	
Loss of protective sensation	39 (100%)
Peripheral Arterial Disease	15 (38·5%)
Toe Brachial Index	0.5 (±0.1)
Laboratory data	
Glycosylated Haemoglobin (HbA1c) (%)	8.5 (±2.5)
Erythrocyte sedimentation rate (mmol/L)	54.3 (±33)
C-Reactive Protein (mg/l)	28.1 (±25)
White Cell Count	9.2 (±2·4)
Infection grading and classification (IDSA)	
Mild	5 (13%)
Moderate	25 (64%)
Severe	9 (23%)
Systemic antimicrobial/route of delivery	
Cephalexin/oral	6 (15%)
Amoxycillin + clavulanic acid/oral	13 (33.5%)
Flucoxacillin/oral	3 (8%)
Clindamycin/oral	1 (2.5%)

Ciprofloxacin/oral	1 (2.5%)
Rifampin + fusidic acid/oral	2 (5%)
Sulfamethoxazole + trimethoprim/oral	1 (2.5%)
Combination therapy/oral	3 (8%)
Piperacillin + tazobactam/Intravenous	6 (15%)
Cephazolin/Intravenous	3 (8%)

16S rDNA next generation sequencing generated 1,028,895 sequences, which were clustered and aligned at 97% similarity to reveal 1,139 unique OTUs. A total of seven major phyla were identified including Firmicutes (48%), Proteobacteria (26%), Actinobacteria (12%), Bacteroidetes (8%), Fusobacteria (2%) and Cyanobacteria (1%). The clustering of OTUs contributing to greater than 10% within each DFU sample at the genera/species-level is noted in Table 3.2 and those contributing to <10% are noted in Table 3.3.

Table 3.2 Microorganisms contributing ≥10% in each DFU sample (representing the dominant taxa)[255]. * refers to the species level identification of Staphylococcus genus level data.

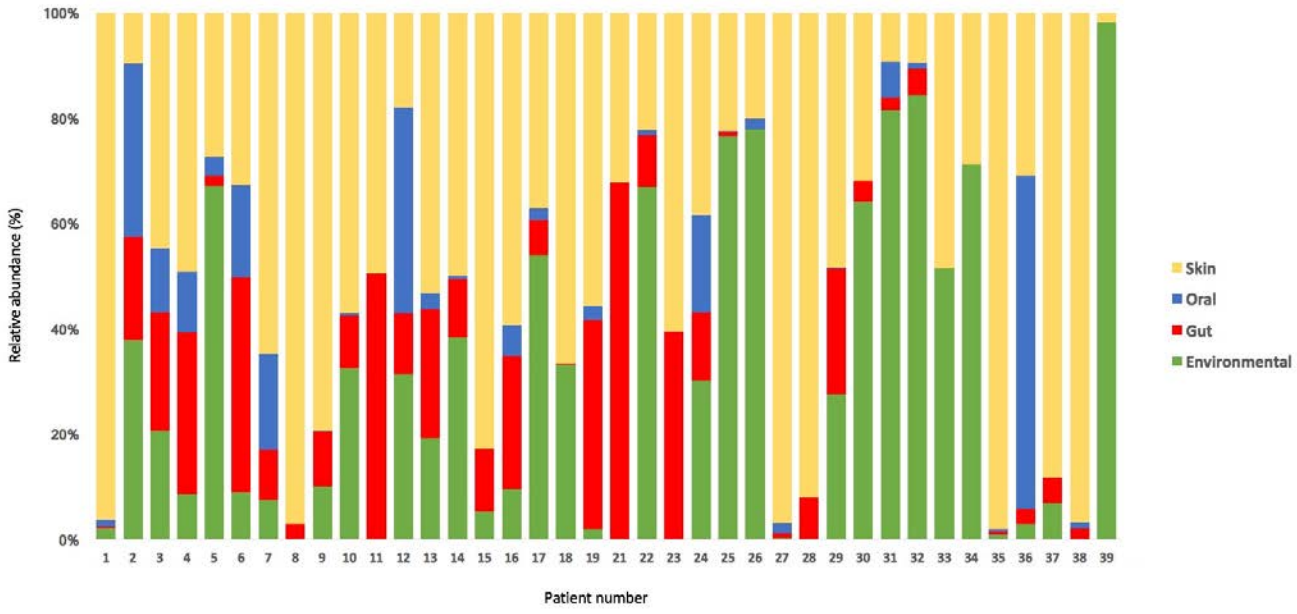
Genera/Species	Samples	Avg abundance %	SD	min-max avg abu %	Aerotolerance
Staphylococcus spp.:	15	40.7	30.3	12 to 92	Facultative
**Staphylococcus aureus*	8	43.1	32.9	12 to 92	Facultative
**Unclassified Staphylococcus* spp.	7	31.8	28.2	12 to 81	Facultative
**Staphylococcus pettenkoferi*	2	26	3	23 to 29	Facultative
Corneybacterium striatum	8	32	16.6	12 to 59	Facultative
Finegoldia spp.	7	12	2.8	10 to 18	Anaerobe
Peptoniphilus spp.	7	14.5	5.1	10 to 22	Anaerobe
Acinetobacter baumannii	7	30.5	18.7	16 to 69	Facultative
Anaerococcus spp.	6	14.3	5.1	12 to 24	Anaerobe
Streptococcus agalactiae	5	45.2	39	16 to 89	Facultative
Enterobacter spp.	5	19.6	8.1	10 to 28	Facultative
Proteus spp.	4	22.7	4.5	19 to 23	Facultative
Prevotella spp.	4	14.3	4	10 to 18	Anaerobe
Haemophilus spp.	4	21	14	12 to 42	Facultative
Blastocatella fastidiosa	3	24	11	12 to 32	Facultative
Pseudomonas aeruginosa	2	12.5	3.5	10 to 15	Aerobe
Porphymonas spp.	2	11.5	2	10 to 13	Anaerobe

Table 3.3 Microorganisms contributing between 1-10% in each DFU sample (representing major contributors) [255]. * Refers to the species level identification of Staphylococcus genus level data.

Genera/Species	Samples	Average abundance %	SD	Range %	Aerotolerance
Corynebacterium spp.	13	3.5	2	1 to 8	Aerobe
Anaerococcus spp.	10	4	2.6	1 to 8	Facultative
Staphylococcus spp.: -	10	2.9	1.6	1 to 6	Facultative
**Staphylococcus epidermidis*	5	2.8	0.9	2 to 4	Facultative
**Staphylococcus xylosus*	3	1.3	0.6	1 to 2	Facultative
**Staphylococcus aureus*	1	1	0	1	Facultative
**Staphylococcus simulans*	1	1	0	1	Facultative
Finegoldia spp.	9	4.4	2.6	1.5 to 8	Anaerobe
Acinetobacter spp.	9	3.8	2	2 to 8.5	Aerobe
Propionibacterium spp.	8	2.5	1.6	1 to 5	Facultative
Cyanobacteria_Subsectionl	7	4.9	2.5	2 to 9	N/A
Cenarchaeum	7	4.4	2.5	1 to 8	N/A
Streptococcus spp.	6	3.6	2.8	1 to 7.5	Facultative
Pseudomonas aeruginosa	6	4	2.9	1 to 7.6	Aerobe
Proteobacteria_ARKDMS-49	6	6.4	1.7	4 to 8	N/A
Porphymonas spp.	6	4	3.4	1 to 9	Anaerobe
Peptoniphilus spp.	6	4.9	3.6	1 to 8.5	Anaerobe
Rhodothermaceae spp.	5	4.7	2.7	2 to 8	N/A
Veillonella spp.	5	1.6	0.4	1 to 2	Anaerobe
Proteobacteria_E01-9C-26 marine	5	6.4	3.1	1 to 9	N/A
Elizabethkingia meniingoseptica	5	4.3	2.1	2 to 7.5	Aerobe
Candidatus Hepatobacter penaei	5	3.7	2.3	2 to 6.5	Anaerobe
Aerococcus spp.	5	2.9	1.5	1.5 to 5	Aerobe

Staphylococcus spp was the most prevalent microorganism in infected DFUs. This was followed by *Corynebacterium* spp., *Finegoldia* spp., *Peptoniphilus* spp., *Acinetobacter* spp., *Anaerococcus* spp., and *Streptococcus* spp., We further categorized microorganisms based on their residing niche (environmental, skin, oral and gut) to better define the site of origin of microorganisms that colonize DFUs (Figure 3.1). Microorganisms commensal to the skin were predominant in half of patients (50.6%) followed by environmental (29.1%), gut (14%) and oral (6.3%) microorganisms.

Figure 3.1 Residing niche of sampled microorganisms identifies skin, environment, gut and oral microbes colonizing DFUs.



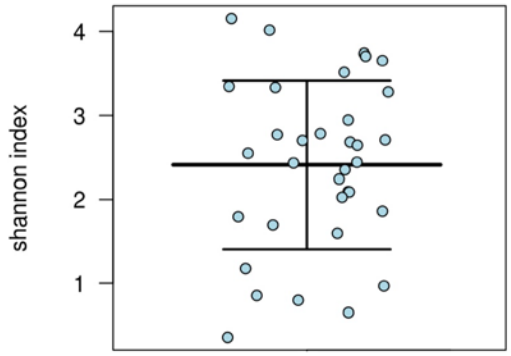
Community structure of DFIs are heterogeneous

The community structures of DFIs were depicted using rarefaction and Shannon Weaver index plots, which explore the richness, and diversity of individual infected DFUs (Figure 3.2).

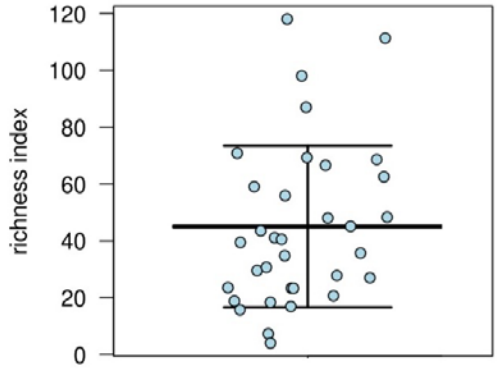
Figure 3.2 Community diversity and richness reported for 39 patients with DFI. (a) Community diversity of DFUs presented using the Shannon-Weaver index at maximum read length of 300. Shannon Weaver Index is a measure of diversity that includes the number of unique microbial taxa and their relative evenness within each sample. Thus, a higher Shannon Weaver Index correlates to a greater diversity. (b)

Community richness of DFUs presented using richness index reporting the number of unique OTUs in each wound sample. Data sets were normalised to remove low abundance OTUs contributing to less than 1% within each wound sample.

(a)

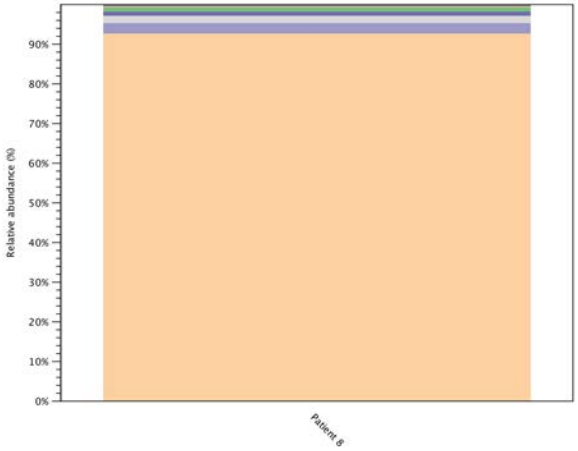


(b)

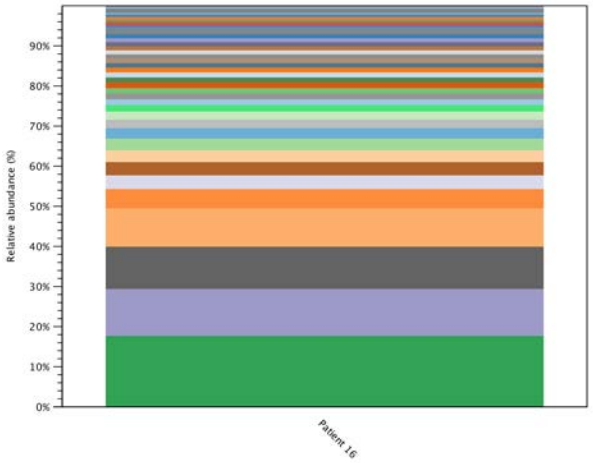


Most DFUs had complex polymicrobial communities with great heterogeneity between patients. Rarefaction identified a mean of 56 OTUs (±31.2, range 4 to 125) per DFU, and Shannon Weaver index identified mean indices of 2.3 (±0.9, range 0.4 – 4.1). Descriptive statistics allowed for a more clinically relevant picture to be composed of the overall community structure. We identified three general profiles that sub-divided DFUs based on their community structure (Figure 3.3). High frequency taxa mostly comprised of a single microorganism (±3) (i.e. monomicrobial infection), High to low frequency taxa were comprised of between one to five (±2) dominant microorganisms followed by many low frequency taxa (i.e. polymicrobial infection) and low frequency taxa comprised on average of ≥20 (±) minor microorganisms (complex polymicrobial infection).

(a) High frequency taxa.



(b) High to low frequency taxa.



(c) Low frequency taxa.

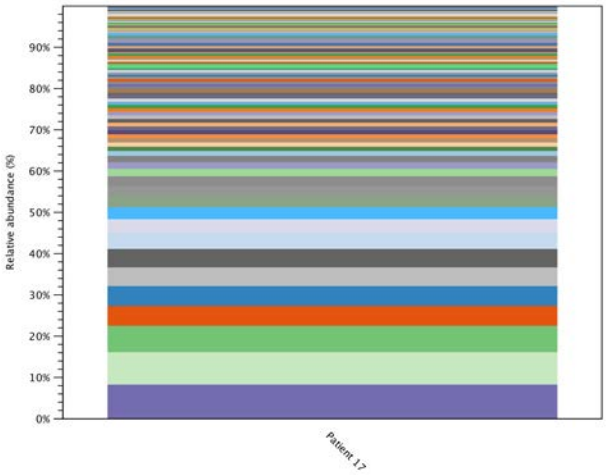


Figure 3.3 Bar chart represent relative abundances (%) of taxa profiles for 39 DFUs. Each bar represents individual genera/species. (a) High frequency taxa were observed in ten patients (26%), mostly comprised of a single microorganism (±3) (i.e. monomicrobial infection) contributing to ≥88% (±5.4%) of total abundance. (b) High to low frequency taxa were the most common profile and were observed in 25 patients (64%). Low frequency taxa comprised on average of ≥20 (±) minor microorganisms each contributing <1%-5% abundance and no single microorganism contributing >10% (complex polymicrobial infection). (c) Low frequency taxa were infrequently observed in only four patients (10%) and contained higher relative abundances of environmental microorganisms ($p < 0{\cdot}01$).

The duration of DFU prior to infection presentation may present a major driver behind the microbiota

Five (13%) DFUs at the time of presentation were less than six-weeks in duration and were composed of high frequency taxa with one predominant microorganism (Figure 3.4). These were *S. aureus* in three cases and *S. algalactaie* in two cases. The relative abundance of *Staphylococcus* spp. was far greater in DFUs < six-weeks than DFUs of longer duration where it was present but at significantly lower relative abundances (Figure 3.5).

Figure 3.4 Bar chart representing relative abundance of taxa in acute diabetic foot ulcers (<6 weeks duration).

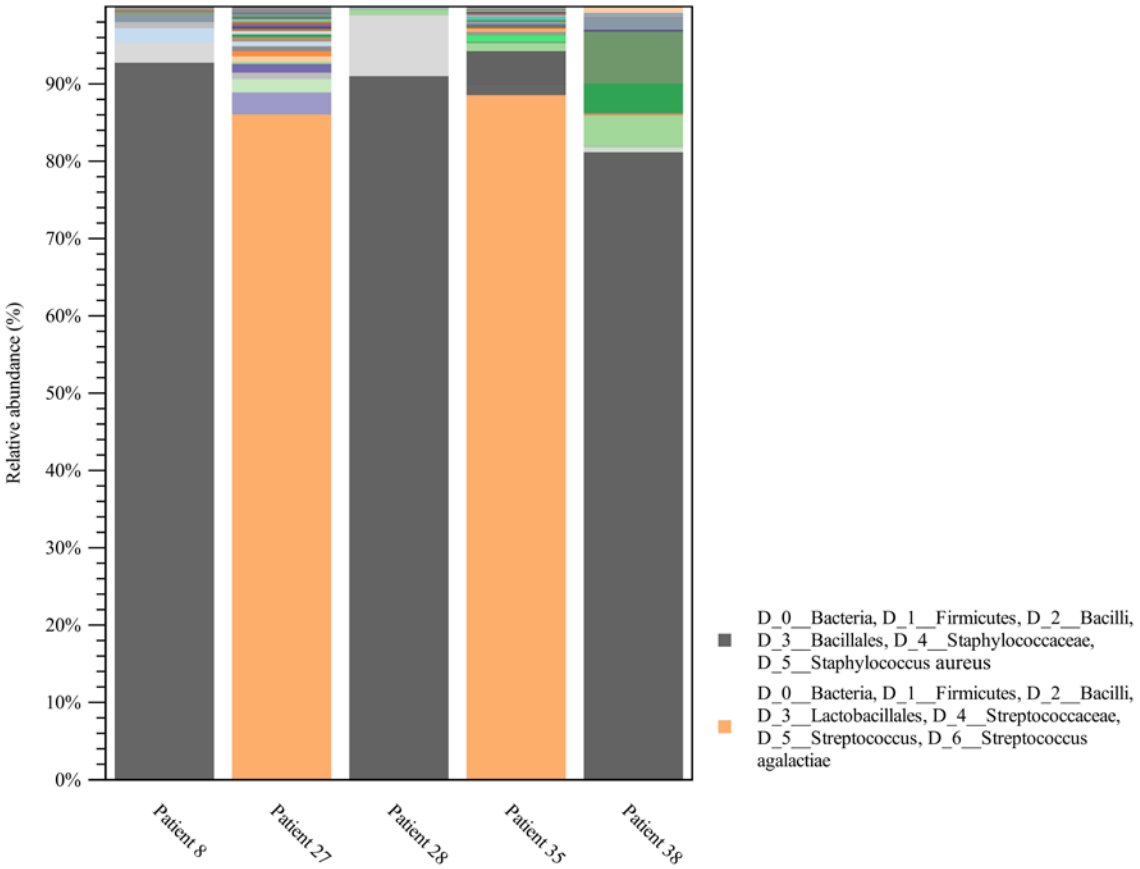
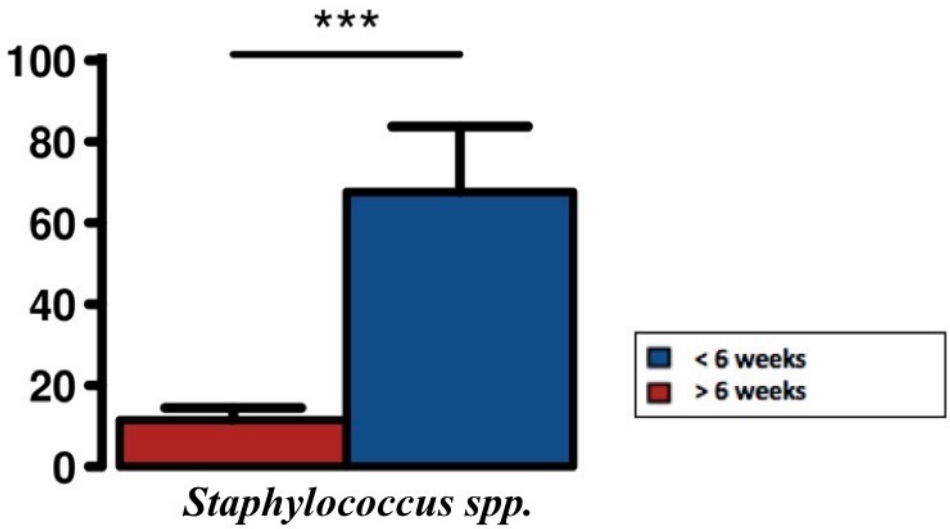


Figure 3.5 Analysis of variance between Staphylococci spp. relative abundance (%) and DFUs based on duration. In DFUs < six weeks, *Staphylococcus* spp. were present as the dominant taxa (high frequency).



Longer duration DFUs (≥6 weeks) with a new acute infective episode (n=34, 87%) were the most common presentation. PCoA Bray-Curtis plots with PERMANOVA identified the community structures between longer and shorter duration DFUs were dissimilar (p = .003) (Figure 3.6 and Figure 3.7).

Figure 3.6 PCoA Bray-Curtis plots identify that differences are present in the community structures between longer and shorter duration DFUs.

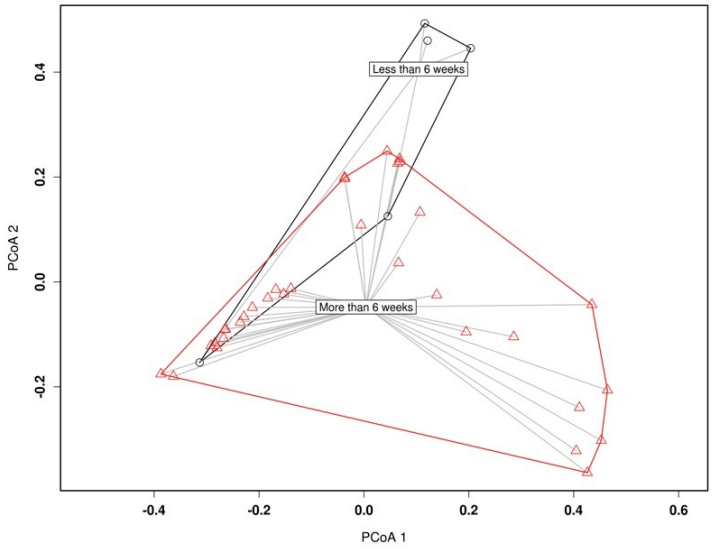
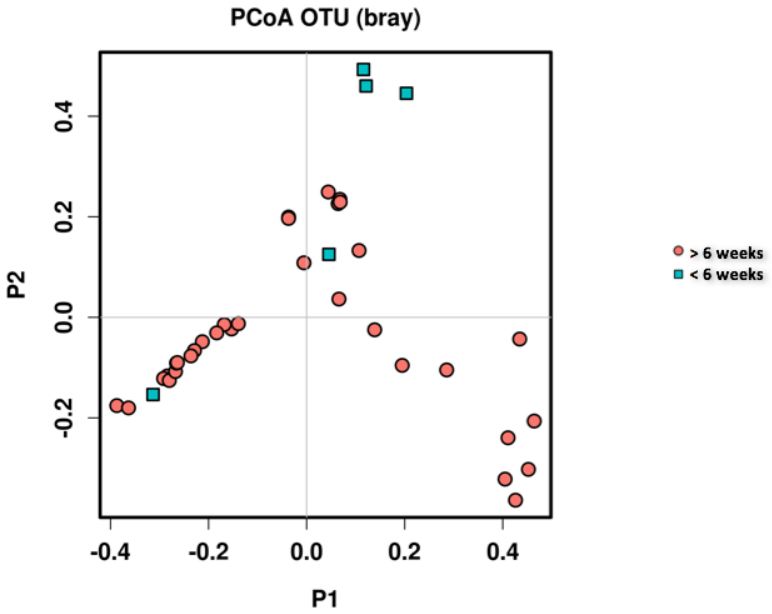
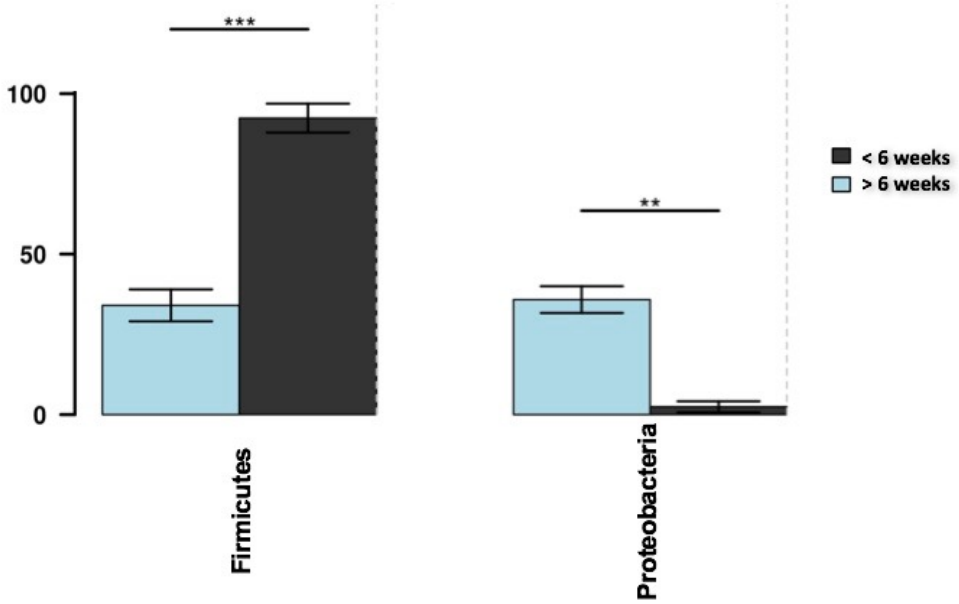


Figure 3.7 PCoA Bray-Curtis plot demonstrates how similar/dissimilar the community structure of DFUs less than 6 weeks.



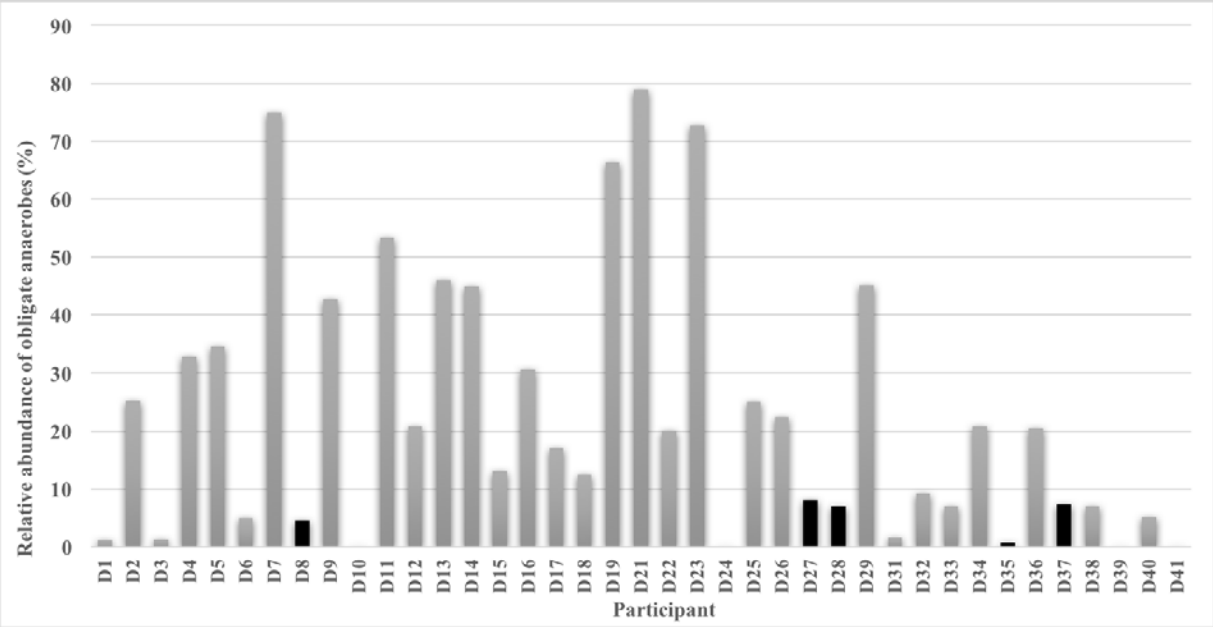
Furthermore, ulcer duration was positively correlated with relative abundance of Proteobacteria ($p < .05$) and negatively correlated with relative abundance of Firmicutes ($p < .05$) (Figure 3.8).

Figure 3.8 Ulcer duration was positively correlated with relative abundance of Proteobacteria ($p < .05$) and negatively correlated with relative abundance of Firmicutes ($p < .05$).



Closer examination of OTUs revealed that *Staphylococcus* spp., contributed to the positive correlation detected between Firmicutes and relative abundance and ulcer duration ($p < .05$). Spearman's rank correlation coefficients further clarified that DFUs of longer duration were polymicrobial, typically having greater number of OTUs and were broader in diversity ($p = .01$). This statistical approach further correlated higher frequencies of DFUs containing obligate anaerobes that constituted greater than 30% of the total abundance in DFUs of greater duration ($p = .03$) (Figure 3.9).

Figure 3.9 Relative abundance (%) of obligate anaerobes at the individual samples level identifies great heterogeneity in thirty-nine patients with infected DFUs. Black bars represent DFUs <6 weeks and grey bars represent DFUs >6 weeks.



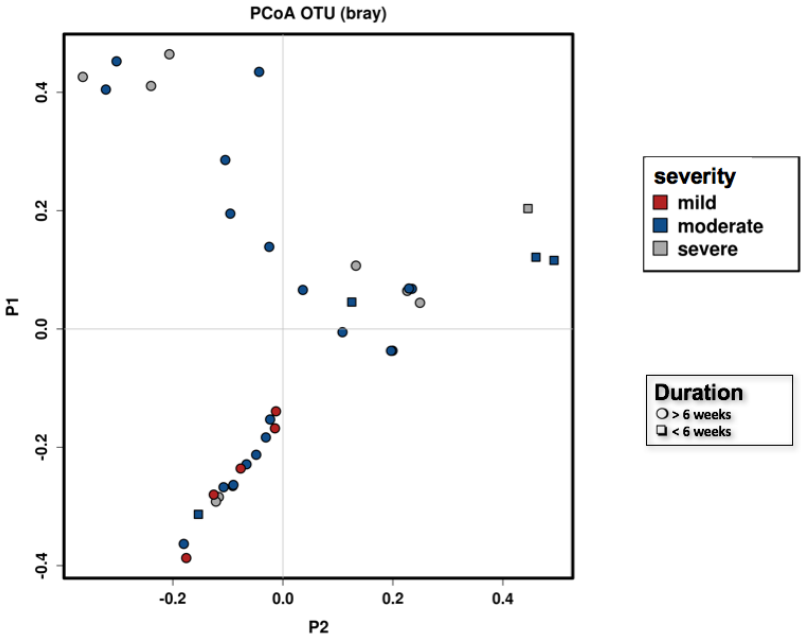
Wound observations and clinical factors lack association with the microbiota

Associations between clinical factors and DFI microbiota were compared using PERMANOVA and Spearman's rank correlation coefficients. The location, depth and the level of glycosylated haemoglobin (HbA1C ≥7%) were not associated to any significant bacteria. The presence of slough or malodour within an infected DFU were independently associated with community structure but were not inversely correlated to each other (p = .7). Slough in an infected DFU was associated with higher abundances of obligate anaerobes (slough present and ≥30% anaerobe present =13 of 39, 33%, p = .01), as was malodour (malodour DFUs =15, mean anaerobe abundance 34%, SD 25.3 versus no malodour of DFUs, mean anaerobe abundance 15%, SD 18.4).

Infection severity of Diabetic Foot Infections are associated with altered community structures

PERMANOVA identified some disparity between the community structure and infection severity. Mild DFIs were different from both moderate infection (p = .01) and severe infection (p < .001) (Figure 3.10) and were positively correlated to fewer OTUs and were less diverse. In contrast, severe infections often presented exclusively with low frequency taxa profiles (n=3 of 4, p = .02). Obligate anaerobes and their abundance within each DFU were explored for relationships between infection severities. The abundance of anaerobes was similar across mild DFIs (abundance= 29.5%, ±31) moderate DFIs (abundance= 20.5%, ±22.3) and severe DFIs (abundance= 27.3%, ±21), indicating that there exist no differences between patients presenting with more severe infections and a greater abundance of anaerobes (p = .6).

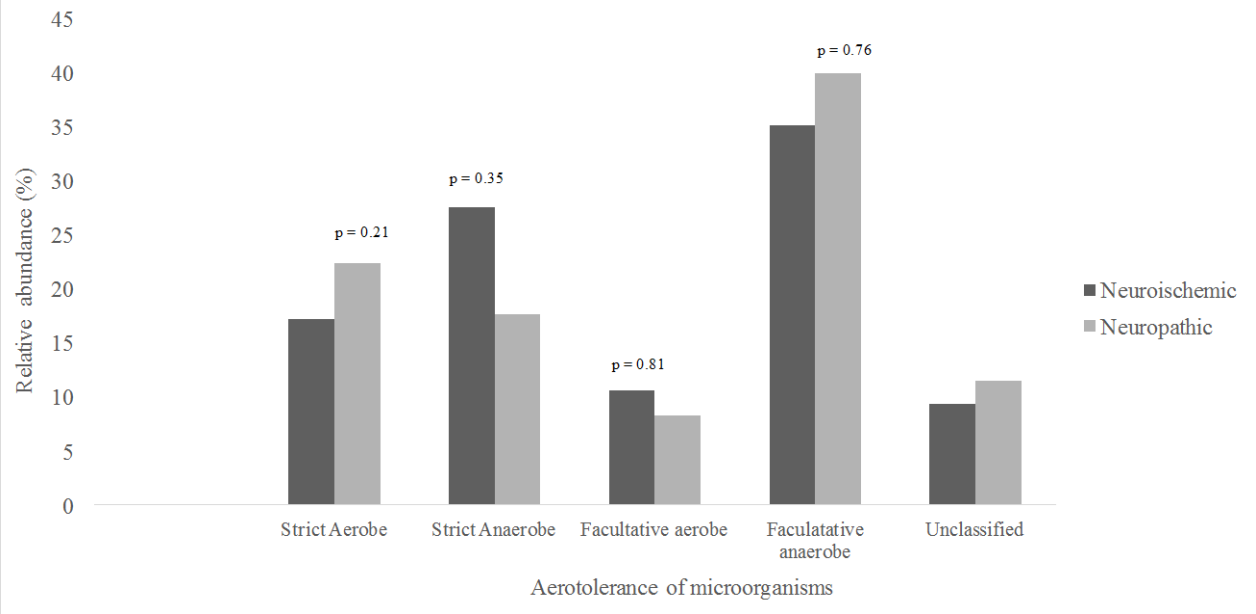
Figure 3.10 PCoA Bray-Curtis plot demonstrates the community structure difference between infection severities in addition to defining the duration of DFU.



Neuropathic and Neuroischemic Diabetic Foot Ulcers harbour similar microbiota's

Twenty-three patients were classified as having neuropathic DFUs (normal TBI ranges ≥0.9 - <1.2 and MNDS≥6) and sixteen patients were classified as having neuroischemic DFUs (TBI <0.7 to 0.3, and MNDS≥6). Non-parametric approaches identified no difference in the mean average abundances between neuropathic and neuroischemic DFUs (Figure 3.11).

Figure 3.11Relative abundance of species of differing aerotolerance in neuropathic (n=25) and neuroischemic (n=16) infected diabetic foot ulcers.



Culture dependent methods underestimate anaerobic microorganisms

Kappa coefficients were used to determine the level of agreement between culture-dependent methods and 16S rDNA next generation sequencing. Agreement in the identification of obligate anaerobes was poor between culture and DNA sequencing (p = .4). Culture underestimated obligate anaerobe presence in 90% of samples (detection of obligate anaerobes by culture = 4 of 39, 10% vs detection of obligate anaerobes by DNA sequencing = 34 of 39, 79%).

Microbial load of DFUs

Microbiological information on the microbial load of DFUs was assessed using both conventional culture (n = 21, mean cfu / gram of tissue 1.67×10^7, SD 4.16×10^8) and qPCR (n = 39, mean DNA copies / gram of tissue 5.92×10^8, SD 1.02×10^9). This identified no statistical difference between the two methods (p = .o8)

Table 3.4 The microbial load of DFUs compared using 16S rDNA sequencing and conventional culture.

16S rDNA sequencing (DNA copies / per gram of tissue)	Conventional Culture (cfu / per gram of tissue)
1.64×10^7	1.1×10^5
3.97×10^7	1.56×10^4
9.69×10^7	5.62×10^5
8.32×10^7	1.67×10^6
5.55×10^8	3.33×10^5
5.27×10^7	3.53×10^7
3.03×10^8	2.50×10^6
4.06×10^7	5.00×10^7
2.26×10^7	6.44×10^6
3.81×10^7	3.25×10^4
1.42×10^8	1.95×10^5
1.16×10^9	8.33×10^5
8.35×10^4	1.74×10^7
2.10×10^7	1.57×10^8
3.89×10^6	1.57×10^8
4.97×10^8	9.50×10^7
1.27×10^8	1.04×10^9

1.18×10^{9}	1.25×10^{7}
1.05×10^{8}	1.92×10^{9}
3.63×10^{6}	8.25×10^{6}
1.35×10^{9}	1.25×10^{6}
6.95×10^{8}	N/A
4.01×10^{9}	N/A
1.46×10^{8}	N/A
1.14×10^{9}	N/A
3.90×10^{8}	N/A
$5.75^{7} \times 10$	N/A
1.13×10^{9}	N/A
6.17×10^{7}	N/A
3.34×10^{8}	N/A
3.44×10^{9}	N/A
2.61×10^{9}	N/A
5.99×10^{7}	N/A
2.12×10^{7}	N/A
6.56×10^{7}	N/A
1.02×10^{8}	N/A
2.21×10^{9}	N/A
6.52×10^{9}	N/A
4.07×10^{8}	N/A
1.63×10^{9}	N/A
1.60×10^{8}	N/A

Treatment parameters and outcomes

In total, nineteen patients (49%) during the study period experienced treatment failure. Of the thirty-three patients who had DFUs >6 weeks, fifteen (45%) with moderate to severe IDSA infections experienced treatment failure. In the group of five patients with DFUs < 6 weeks, four patients (80%) with moderate IDSA infections experienced treatment failure. These infections were mono-microbial and were predominated by high frequency taxa of either *Staphylococcus* spp. or *Streptococcus* spp. Correlation coefficients were used to explore if DFUs containing high relative abundances of commonly cited pathogens of infection in DFI (*S. aureus, S. agalactiae* and *A. baumannii*) were at greater risk of treatment failure. This revealed the presence of *S. agalactiae* in DFUs (regardless of duration of DFU) were associated with greater treatment failures (p = .007).

PERMANOVA revealed no further differences in the community structures between patients who failed therapy and those who experienced treatment success (p = .2). In patient samples where obligate anaerobes were identified as contributing to the overall wound microbiota at levels greater than both 30% and 50%, there were no increased tendency towards failing therapy. The type of anti-infective therapy provided to patients in this study provided adequate anaerobe cover (25 of 39 wounds received antimicrobials with anaerobe cover, 64%) and this may explain the lack of significance between high relative abundance of anaerobes in DFUs and no increased tendency to fail therapy.

Thirteen patients (33.3%) received narrow spectrum antimicrobials with nine (23.1%) of these patients having DFUs >6 weeks duration. Four of these nine patients (44.4%) experienced treatment failure whilst receiving narrow spectrum antimicrobials, but the five remaining patients with DFUs > 6 weeks on narrow spectrum antimicrobials experienced treatment

success. The number of cases were too low for statistical analysis. Twenty-five patients received broad-spectrum antimicrobials with eleven patients (44%) experiencing treatment failure. There were no correlations between a tendency to fail therapy and being on either narrow spectrum or broad-spectrum antimicrobials. A Generalised Linear Model (GLM) was performed to identify any predictors of treatment failure independent of the microbiota. These identified patients having a low TBI (<0.7) as being the only predictor of failure regardless of the microbiota (p = .01). No other clinical factors such as a level of glycosylated haemoglobin greater than ≥7% (p = .72) or the severity of infection were correlated to treatment failure (Mild, p = .13, Moderate, p = .65, Severe, p = .26).

3.1.4. DISCUSSION

In the context of managing diabetic foot infections from an infectious disease viewpoint, current guidelines based on culture-dependent data are now subject to the scrutiny of molecular DNA based approaches. Of studies employing amplification and sequence analysis of the 16S rDNA to characterize the microorganisms involved in DFI, none to date have sampled participants with overt clinical signs of infection. Instead the available data report on chronic, new or recurrent DFUs that are clinically non-infected. Given the increasing utilisation of genomic analysis from both a clinical and research domain, it is essential to understand the microbiome of clinically infective DFUs and if current anti-infective practices can be improved through the translation of complex bioinformatics arising from the DNA analysis of microbial communities. We analysed robust microbiome datasets from infections of the feet in people with diabetes, and detailed their clinical outcomes, relating this back to current anti-infective practices. The duration of a DFU prior to presenting with a new clinical infection may help clinicians decide on the antimicrobial regimen of choice.

We identify *Staphylococcus* spp., as the most commonly sequenced dominant bacteria in approximately one third of DFUs in this study, followed closely by *Corynebacterium* spp. In a recent review by our group on the bacteriology of DFUs from both a molecular and culture based approach [325], the predominant pathogen/s of infection for DFI was *S. aureus*. *Corynebacterium* spp., *Streptococcus* spp., and obligate anaerobes belonging to *Clostridiales* family XI, all identified as major players in this study, were similarly identified in studies of chronic non-infected wounds. Based on our molecular data and that of previous molecular and culture-based publications, current guidelines that promote the use of antimicrobials targeting Gram-positive aerobic cocci as a first line treatment are appropriate.

Corynebacterium spp. have provided a continuing source of debate regarding their role as a non-pathogenic skin commensal [42] or as a pathogen of infection in the presence of an immunocompromised patient [9,326]. In this study, we seldom identified the presence *Corynebacterium* spp. as a sole pathogen (High frequency taxa), almost exclusively occurring in combination with other known pathogens of DFI. This suggests that there may be a role for *Corynebacterium* spp. as part of a polymicrobial infection. Given that many first line antimicrobials of choice for DFI are active against this species of microorganism, there may not be a requirement to target this microorganism unless a mono-microbial culture is identified.

Community structure is essentially the composition of a community, including the number of species in that community, their relative numbers (Richness) and their complexity (Diversity). We identify that the duration of DFU is a major driver behind the microbiota, with longer duration DFUs typically having greater species richness and diversity. This correlates to increased relative abundances of Gram-negative Proteobacteria and reduced relative

abundances of Firmicutes in a pattern previously described by Gardener et al on neuropathic non-infected DFUs [10]. Proteobacteria are commonly identified in wounds [8,90] and largely belong to the *Pseudomonadaceae and Enterobacteriaceae* families. It is unclear from our data if these microorganisms require special attention. For example *P. aeruginosa* was present as minor taxa in only one quarter of samples (8/25 DFUs), thus supporting the general consensus [2] that *P. aeruginosa* is not a typical pathogen of infection in DFI (excluding southern hemisphere locations) [327], and may not require additional tailored therapy should it be identified through cultivation-based methods.

Obligate anaerobes were also identified in 90% of DFUs, but great heterogeneity existed between patients with regards to their relative abundances. In most DFUs, obligate anaerobes co-existed in high abundances with aerobic microorganisms, suggesting that obligate anaerobes likely play a role as co-pathogens in DFI. Current microbiology laboratories do not employ culturing methods to isolate many of the obligate anaerobes identified in this study through DNA sequencing. However, even in the absence of culture-dependent guidance, many commonly utilised antimicrobials for DFI are active against obligate anaerobes.

Furthermore, there are no studies exploring if additional anti-anaerobic therapy improves DFI outcome, and in this study, we find no correlation between the high relative abundance of obligate anaerobes and a greater tendency to fail antimicrobial therapy. The decision to use targeted antimicrobials against obligate anaerobes by clinicians should be administered under the guidance of antimicrobial stewardship [328]. The pattern of antimicrobial therapy prescribing in this study were based on specialist Infectious Disease physicians with experience of managing these complex patients, but these results may reflect differently when managed by

non-specialist clinicians with limited exposure to these wounds. Further work in this area is required.

The current guidance materials available to clinicians managing DFIs are predominantly based on culture-dependent data, yet this study employing DNA sequencing techniques re-enforces most of this data as being clinically relevant [2,25]. Pyogenic cocci were predominant in acute DFUs in this study, and this finding supports directed narrow spectrum antimicrobial regimens (with consideration for culture sensitivities looking for MRSA). DNA-sequencing methods however, highlight the limitation of conventional bacterial cultures with regards to the microbial diversity and ability to isolate microorganisms not detected under routine clinical microbiology laboratory protocols. Many of these difficult to isolate microorganisms were found in chronic wounds that harboured flora similar to the environment, suggesting patients expose their wounds to an array of environments (i.e. barefoot walking, showering with no wound dressing, gardening etc.). Therefore, patient education is vital in order to minimize exposure of DFUs to environmental contaminants and opportunistic pathogens.

Current guidelines for classifying and managing infected DFUs provide guidance (in conjunction with local policies and patterns of microbial sensitivities for resistance) on the duration and route of delivery of antimicrobials based on infection severity [2,25]. The use of broad-spectrum antimicrobials delivered parenterally is promoted for severe DFIs, owing to the polymicrobial nature of infection. We confirm severe DFIs are extremely diverse, polymicrobial and complex, and our data supports current clinical practice by parenteral, broad-spectrum antimicrobials is warranted. Exploration from a larger sub-set of patients with severe DFI composing of low frequency taxa profiles, may provide greater insight into managing these challenging infections.

Previous reports have suggested that DFUs complicated by peripheral arterial disease (i.e. ischemic or neuroischemic) likely lead to an altered wound microenvironment and thus microbiota [10]. Additionally, the presence of peripheral vascular disease as a comorbid variable in the presence of an infected foot in a person with diabetes has been has been reported as an independent predictor of poor outcome [329,330]. Sixteen patients in this study had neuroischemic ulcers (TBI <0.7 to 0.3) with most DFUs presenting with mild to moderate peripheral arterial disease.

We ascertained that both neuropathic and neuroischemic (patients with mild to moderate PAD) DFUs harbour similar microbiota's and the requirement to segregate these differing wound aetiologies may not be required for microbiome analysis when using a TBI cut off value of 0.5 as an arbitrary marker.

Nineteen patients during the study period experienced antimicrobial treatment failure, but no differences existed in the microbiota of patients who failed therapy and those who experienced treatment success. Furthermore, patients who were treated with either narrow spectrum or broad-spectrum antimicrobials experienced similar failure rates (44.4% versus 44%) and this suggests that other factors are likely at play including the host immune response to infection, patient compliance in adhering to therapy and or peripheral perfusion. A general linear model approach identified that the presence of a TBI <0·7 was an independent predictor of treatment failure regardless of the microbiome, or antimicrobial, emphasising the difficulties in managing these complex infected wounds.

Whilst our microbiome data identifies DFUs of greater than six-weeks duration presenting with a new clinical infection (includes mild-moderate-severe, with no discrimination) are often

polymicrobial, with exception to nearly always targeting aerobic Gram-positive cocci, the requirement to also target anaerobes requires further research. Furthermore, whilst DNA sequencing provides an extended view of the microbiota, it is limited in providing information on "which microorganisms" maybe directly contributing to infection.

This is increasingly important when analysing our data set where regardless of the spectrum of activity of antimicrobials (i.e. Narrow or broad-spectrum), patients experienced similar outcomes. It may be possible in a highly diverse microbiota, that narrow spectrum antimicrobials targeting pyogenic cocci alone, is enough to reduce the virulence/pathogenicity of infective symptoms without the requirement to use a shotgun approach to target everything broadly. The use of metagenome sequencing may allow us to better understand this question [325].

This paper provides a useful insight into the bacterial communities in infected DFUs and reflects on treatment outcomes of anti-infective therapy and if molecular based data would have altered therapeutic regimens. The paper is limited by the sample size and thus recommendations based on molecular data are not possible. A larger cohort of patients would provide greater detail and where possible analysis of small subsets of interest. This paper also identifies the difficulties with obtaining species level data when using the 16S rDNA. Furthermore, what is strikingly apparent from our data is that whilst we provide an extended view of "which microorganism/s" are present, we cannot be definitive on "which microorganism/s" are responsible as contributing as pathogens of infection. The era of "metagenomics" that can analyse genes responsible for virulence or pathogenicity may unveil these answers.

CHAPTER 4

4.1 MICROSCOPY VISUALISATION CONFIRMS MULTI-SPECIES BIOFILMS ARE UBIQUITOUS IN DIABETIC FOOT ULCERS

APPENDIX 5 & 6. STUDY PROTOCOL AND ETHICS APPROVAL

APPENDIX 8. JOHANI, K., **MALONE, M.,** JENSEN, S.O., GOSBELL, I. B., DICKSON, H. G., HU, H. AND VICKERY, K. MICROSCOPY VISUALISATION CONFIRMS MULTI-SPECIES BIOFILMS ARE UBIQUITOUS IN DIABETIC FOOT ULCERS. *INTERNATIONAL WOUND JOURNAL* 2017. DOI:10.1111/IWJ.12777.

Candidate contributions:

- ✓ Worked as senior/corresponding author for publication
- ✓ Designed the study protocol and methodology, and performed all aspects of clinical study, including screening, recruitment, data collection, tissue biopsy sampling and storage.
- ✓ Used genomic software to quality control and analyse DNA sequences from raw files.
- ✓ Used statistical software and knowledge of statistical approaches to analyse / correlate clinical data.
- ✓ Reviewed all SEM and FISH pictures to confirm +ve presence
- ✓ Wrote the manuscript in full
- ✓ Submitted the manuscript as senior/corresponding author

Abstract

There are many facets to diabetic foot disease that contribute to the development of a DFU, in particular the “triad” of factors that are peripheral neuropathy, peripheral vascular disease and trauma [1]. Foot ulceration leaves a physical break in the protective barrier of the skin where various invading microorganisms may colonize the wound. In a person with diabetes, a retarded immune response is common and this may predispose an ulceration to further microbial invasion and replication, resulting in damage to host tissues and an inflammatory response that is characterized as clinical infection [2].

In some patients who receive optimal standards of care (these include offloading, regular sharp debridement and re-vascularization), and in the absence of overt clinical infection, failure of the DFU to heal cannot be explained. Increasing evidence within the literature has identified the potential of biofilms to complicate wounds and cause a delay to wound healing. This research examines DFUs to ascertain the presence of biofilm, determine their prevalence, explore the microbial diversity and ascertain if clinical cues are useful in detecting wound biofilm. DFU specimens were obtained from 65 subjects. Scanning electron microscopy (SEM) and Fluorescent in situ Hybridisation (FISH) techniques were used to visualize biofilm structures.

16S rDNA next generation sequencing were performed to explore the microbial diversity. Clinical cues that included the presence of slough, excessive exudate, a gel material on the wound bed that reforms quickly following debridement, poor granulation and pyocyanin were correlated to microscopy results. Of the 65 DFU specimens evaluated by microscopy, all were characterized as containing biofilm (100%, $p < .001$). Molecular analyses of DFU specimens

revealed diverse polymicrobial communities. No clinical correlations were identified in aiding clinicians identify wound biofilm.

4.1.1. INTRODUCTION

Foot ulceration is a physical break in the protective barrier of the skin allowing colonization by invading microorganisms. In a person with diabetes, an impaired immune response is common and this may predispose an ulceration to microbial invasion, with resultant damage to host tissues and an inflammatory response that is characterized as clinical infection [2]. In some patients who receive optimal standards of care (including off-loading, regular sharp debridement and re-vascularization), and who do not exhibit overt clinical infection, failure of the DFU to heal might be explained by the presence of biofilm. Planktonic microorganisms that are responsible for acute infections may be readily identified through cultivation-based approaches, while multi-species sessile communities of microorganisms or biofilms may not be detected by the same cultivation methods.

There is also a lack of diagnostic tests to define the presence of wound biofilm, and there are no quantifiable biomarkers. To augment clinical practice, some clinicians have used what they believe are “clinical cues” of biofilm presence through naked eye observations [166,331,332]. Such signs have included; a shiny, translucent, slimy layer on the non-healing wound surface, the presence of slough or fibrin, and gelatinous material reforming quickly following removal in contrast to slough and other devitalised tissue or fibrin that often takes longer to reform. As biofilms are microscopic in nature, doubt has been expressed as to whether biofilms can be visually observed by clinicians. Unfortunately, chronic wound clinical observations have not

been cross-correlated to microscopy approaches, which are better suited for defining the presence of biofilm.

The primary objective of this study was to visualize and confirm the presence of biofilm in DFUs and better understand if they consist of mono or multi-species biofilms. Secondary objectives were to ascertain if commonly used clinical cues were accurate in detecting wound biofilm. SEM, FISH / PNA-FISH and 16S rDNA next generation sequencing was utilized to answer these objectives.

4.1.2. METHODS

Patient population

In this prospective study, 65 consecutive patients aged over 18 years presenting to the Liverpool Hospital High Risk Foot Service with a DFU were enrolled over a six-month period. Individuals were eligible for the study if they had either a DFU that had not responded to standard care and were not healing within an appropriate timeframe (i.e. chronic DFU), or presented with a DFU (acute or chronic DFU) and a new acute clinical infection as defined by the Infectious Disease Society of America Guidelines for Diabetic Foot Infection [333]. Tissue biopsies were obtained from the wound edge for each participant (1 sample per patient) after cleansing the wound with NaCl 0.9%. Clinical observations of DFUs were recorded for each patient. Ethics approval for this study was granted by the South West Sydney Local Health District Research and Ethics Committee (HREC/14/LPOOL/487, SSA/14/LPOOL/489).

Specimen collection, storage and 16S rDNA Next generation DNA sequencing workflows

Specimen collection, storage and the work flows for performing DNA extraction and next generation DNA sequencing were performed as previously described [334].

Fluorescent in situ hybridisation (FISH) and Peptide nucleic acid-based fluorescence in situ hybridization (PNA-FISH)

Biopsy material was embedded in optimal cutting temperature (OTC) embedding matrix (Fisher Scientific, Waltham Massachusetts, United States), frozen at –80ºC, cryo-sectioned to a thickness of 6 μm and mounted on SuperFrost Plus slides (Menzel-Glaser, Lomb Scientific, Australia). Different types of probes were utilized for in situ hybridization as previously described by Thurnheer *et al* (2004) [335]. The choice of specific probes was based on DNA sequencing results that allowed identification of the major genera/species of interest to target. The genus-specific probe; Cy3 labelled *Staphylococcus* spp., probe (final concentration 5 ng/µl)[336], Fluor 488 labelled *Pseudomonas* spp., specific probe (final concentration 20ng/µl) and a universal bacterial probe; Fluro 488 or Cy3, (final concentration 5 ng/µl) [337] were employed. For PNA-FISH, probes and kits were sourced commercially (AdvanDx, Inc., Woburn, MA) using previously described methods[13].

Briefly, species-specific *Staphylococcus aureus*/coagulase-negative *Staphylococci* (CNS) probes were used in conjunction with a universal bacterial probe. The hybridization solution, was added drop wise to each tissue section and hybridized at 55°C for 90 min. The slides were washed for 30 min at 55°C in wash solution. Once dry, the coverslip was mounted using a single drop of mounting medium. Slides were examined using CLSM (Zeiss Axio Imager Microscope and/or ZEISS LSM 880, Carl Zeiss Ltd., Herefordshire, UK). Images were processed using ZEISS ZEN Imaging Software (black edition) and Imaris v 8.4, ImarisXT, Bitplane.

Scanning Electron Microscopy (SEM) and image interpretation

DFU biopsy samples were fixed in 3% glutaraldehyde, followed by 3 washes of 0.1M phosphate buffer prior to serial ethanol dehydration and hexamethyldisilazane incubation (Polysciences, Inc., Warrington, Pa.) as described previously [338]. Dried samples were coated with 20-nm gold film in a sputter coater and examined in a scanning electron microscope. Each sample was scored based on the amount of bacteria/biofilm observed using an arbitrary five-point scale as previously reported [339]. Each tissue sample (average of 3 pictures per sample) was viewed under SEM averaging two hours per sample. Tissue was screened for microbial aggregates and extracellular polymeric substances (EPS) from the wound surface downwards, working in a zigzag pattern at magnifications ranging from 300X to approximately 5,500X. Each sample was scored based on the amount of bacteria/biofilm observed using an arbitrary five-point scale as previously reported [78]. Score 0 = no bacteria observed; score 1 = single individual cells; score 2 = small micro-colonies (~ 10 cells); score 3 = large micro-colonies (~100 cells); score 4 = continuous film; score 5 = thick continuous film.

Characterization and visualization of DFU biofilm

The presence or absence of biofilms in DFUs were confirmed through SEM or FISH / PNA-FISH. For the purpose of the study, the definition of biofilm was "microbial aggregates surrounded by a self-produced or host derived matrix adhering to natural or artificial surfaces in the host, or aggregates associated with but not directly adherent to the surface" [93].

Clinical wound observations

Wound observations at the time of presentation were collected based on previous assumptions for "clinical cues" relating to the presence of biofilm. These were; the presence of slough,

excessive exudate, poor quality granulation tissue, presence of pyocyanin, gelatinous material of the wound surface, gelatinous material reforms quickly.

Statistics

Data relating to the presence or absence of DFU biofilm and clinical wound observations were tested using non-parametric methods (binomial probabilities). The hypothesis for the presence of biofilm was based on a previous report that 60% of chronic wounds have biofilm [12], and this was set as the expected proportion. For clinical wound observations, no previous data were available. Expected proportions were set at 50% i.e. no more than chance alone of clinical wound observations being positive when biofilm positive through microscopy. Data were analysed through Statistical Package for Social Sciences Version 23 (SPSS Inc., Chicago, Illinois, USA). For all comparisons and modelling, the level of significance was set at $p < .05$. Data are given as mean, median and standard deviation (±).

4.1.3. RESULTS

Over a twelve-month study period, 65 consecutive patients with DFUs were recruited. Study demographics are reported briefly. Most patients were male (49, 74.2%) whilst there were 17 females (25.8%) patients. The mean age of study subjects was 58.5 years (±12.3). Type 2 diabetes predominated (type 2 =58, 87.9%, type 1=7, 10.6%), and the mean duration of diabetes was 13.9 years (±7.3). Clinically infected DFUs were present in forty patients (60.6%). These were subdivided by duration of the DFU prior to the development of a new acute infective episode; short duration DFU (<6 weeks) with new acute infection (7, 17.5%), chronic DFU >6

weeks with new acute infection (33, 82.5%). The remaining patients with DFUs (26, 39.4%) were classified as chronic non-healing with no acute clinical infection.

Microscopy analysis

The presence of biofilm was visualized and confirmed in all samples (65 of 65, 100%) using either SEM, FISH / PNA-FISH or both ($p < .0001$) (Table 4.1).

Table 4.1 Biofilm analysis and DFU location, wound duration in weeks and whether infected or non-infectedPresence (+) or absence (-) of biofilm as determined by SEM and FISH analysis, degree of biofilm infection (score) and predominant species identified using 16S rDNA sequencing. NS refers to the inability to obtain an additional sample for microscopy from that patient.

Patient number	Biofilm (+ or -)				Wound metrics		
	SEM	Score	FISH / PNA FISH	Biofilm Diversity	Location of DFU	Duration of DFU (weeks)	Infection status
S01	+	4	+	Multi-species only	Plantar metatarsal head	8	Infected
S02	+	5	+	Mono-species and multi-species	Plantar metatarsal head	16	Infected
S03	+	4	NS	NS	Plantar metatarsal head	36	Infected
S04	+	4	+	Mono-species and multi-species	Plantar metatarsal head	14	Infected
S05	+	3	NS	NS	Plantar metatarsal head	24	Infected
S06	+	4	NS	NS	5th toe dorsal	12	Infected
S07	+	4	+	Multi-species only	Plantar metatarsal head	72	Infected
S08	NS	NS	+	Mono-species only	4th toe dorsal	6	Infected
S09	+	4	+	Multi-species only	2nd toe apex	8	Infected
S10	+	3	NS	NS	Heel	20	Infected
S11	+	4	NS	NS	Heel	12	Infected
S12	+	3	+	Mono-species and multi-species	Plantar metatarsal head	24	Infected
S13	+	4	+	Mono-species and multi-species	Plantar metatarsal head	6	Infected
S14	+	4	NS		Plantar metatarsal head	20	Infected
S15	NS	NS	+	Multi-species only	4th toe apex	12	Infected
S16	NS	NS	+	Mono-species and multi-species	Hallux	8	Infected
S17	+	4	+	Multi-species only	Hallux	26	Infected
S18	+	4	+	Mono-species only	Plantar metatarsal head	32	Infected
S19	-	0	+	Mono-species and multi-species	Hallux	12	Infected
S20	+	3	+	Multi-species only	2nd toe apex	16	Infected
S21	+	5	+	Mono-species and multi-species	Medial Hallux	8	Infected
S22	NS	NS	+	Mono-species and multi-species	Medial Hallux	18	Infected
S23	NS	NS	+	Mono-species and multi-species	Heel	12	Infected
S24	+	4	NS	NS	Heel	24	Infected
S25	NS	NS	+	Multi-species only	Hallux apex	9	Infected
S26	+	5	+	Multi-species only	Plantar midfoot	3	Infected

S27	+	5	+	Mono-species and multi-species	Plantar metatarsal head	3	Infected
S28	+	5	NS	NS	Plantar metatarsal head	6	Infected
S29	+	5	NS	NS	Plantar midfoot	52	Infected
S30	+	4	NS	NS	Plantar midfoot	30	Infected
S31	+	5	NS	NS	Plantar metatarsal head	3	Infected
S32	+	5	NS	NS	Plantar metatarsal head	5	Infected
S33	+	4	+	Mono-species and multi-species	Heel	5	Infected
S34	+	5	+	Multi-species only	Heel	12	Infected
S35	+	5	+	Mono-species and multi-species	Heel	6	Infected
S36	+	4	NS	NS	Plantar metatarsal head	12	Infected
S37	+	4	NS	NS	Hallux	9	Infected
S38	+	4	+	Mono-species and multi-species	Hallux	8	Infected
S39	+	4	+	Multi-species only	Hallux	12	Infected
S40	+	4	NS	NS	Heel	72	Non -infected
S41	+	4	NS	NS	Plantar metatarsal head	40	Non -infected
S42	+	4	+	Mono-species and multi-species	Heel	6	Non -infected
S43	+	4	NS	NS	Plantar metatarsal head	24	Non -infected
S44	NS	NS	+	Mono-species and multi-species	Plantar metatarsal head	12	Non -infected
S45	+	4	+	Mono-species and multi-species	Heel	36	Non -infected
S46	+	5	+	Mono-species and multi-species	Plantar metatarsal head	72	Non -infected
S47	+	5	+	Multi-species only	Heel	7	Non -infected
S48	-	1	+	Multi-species only	Plantar midfoot	28	Non -infected
S49	+	4	+	Multi-species only	Heel	18	Non -infected
S50	+	5	+	Multi-species only	Heel	28	Non -infected
S51	+	4	+	Mono-species and multi-species	Plantar metatarsal head	27	Non -infected
S52	+	5	+	Mono-species and multi-species	Plantar metatarsal head	28	Non -infected
S53	+	4	NS	NS	Hallux	6	Non -infected
S54	+	5	+	Mono-species and multi-species	Hallux	6	Non -infected
S55	+	4	+	Mono-species and multi-species	Heel	6	Non -infected
S56	+	3	+	Multi-species only	Plantar metatarsal head	16	Non -infected
S57	+	5	+	Multi-species only	Heel	20	Non -infected
S58	+	5	+	Mono-species and multi-species	Lateral leg	14	Non -infected
S59	+	4	+	Multi-species only	Heel	10	Non -infected
S60	+	5	+	Mono-species and multi-species	Plantar metatarsal head	27	Non -infected
S61	+	4	+	Multi-species only	Hallux	8	Non -infected
S62	+	4	+	Mono-species and multi-species	Plantar metatarsal head	12	Non -infected
S63	+	5	+	Mono-species and multi-species	Hallux	6	Non -infected
S64	+	5	NS	NS	Heel	52	Non -infected
S65	+	5	+	Mono-species and multi-species	Plantar metatarsal head	9	Non -infected

Multiple images (minimum of three images per tissue sample) were viewed under microscopy for each sample to provide an overall score. SEM images identified a predominance of coccoid cells, which often appeared to be coated with EPS (Figures 4.1 – 4.4). When scoring samples, the majority had large micro-colonies (~100 cells) plus the presence of continuous or thick film of extracellular matrix i.e. a score of 4 (52%) or 5 (36%,). Biofilm presence was negative in two samples by SEM (S19 and S48), and a further seven SEM samples were not obtained due to inadequate amounts of DFU tissue. In the absence of SEM, all samples were positive using PNA-FISH with CLSM. DFUs were further sub-categorised for biofilms structures based on their duration, with most samples coming from chronic DFUs (>6 weeks with or without infection, 60 of 65, 92%). Five DFUs of short duration (<6 weeks) with clinical infection were also visualized as containing biofilm.

Figure 4.1 Scanning electron micrograph of DFU obtained from four patients demonstrating biofilm structure. Scanning electron micrograph of DFU obtained from four patients demonstrating biofilm structure. Red arrow identifies large micro-colonies of predominantly coccoid microbial cells encased in thick extracellular matrix (EPS) and anchored to collagen bundles within the wound (biofilm score 5).

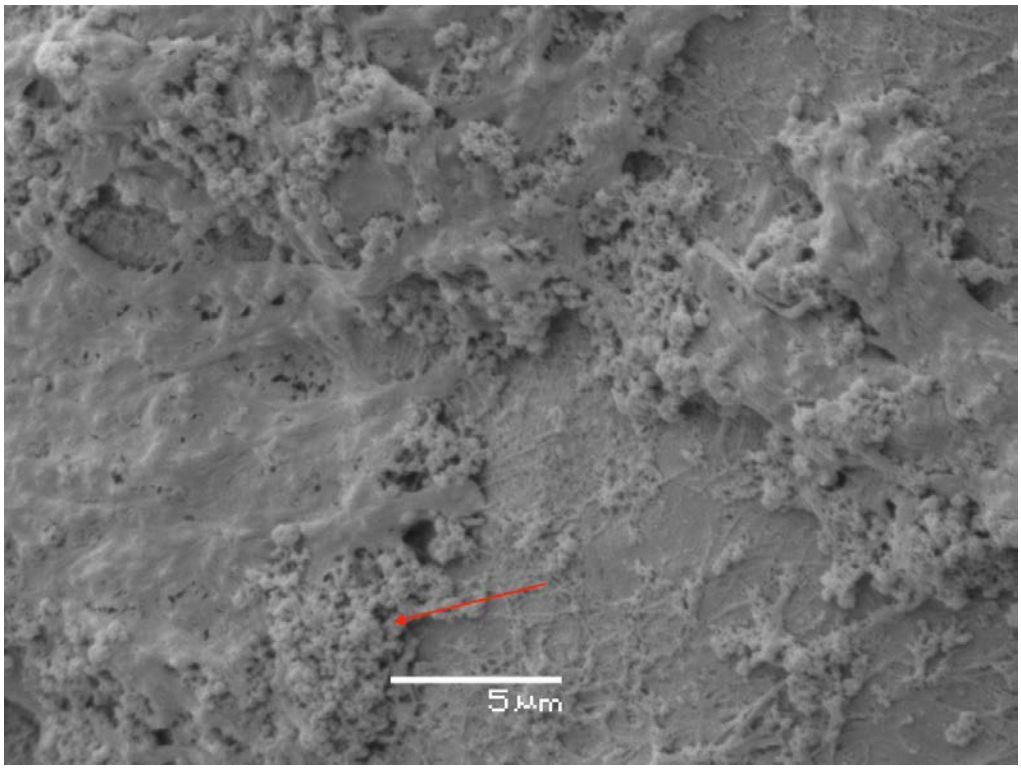


Figure 4.2 Identifies large micro-colonies of predominantly coccoid microbial cells encased in thick extracellular matrix (EPS) and anchored to collagen bundles within the wound (biofilm score 5).

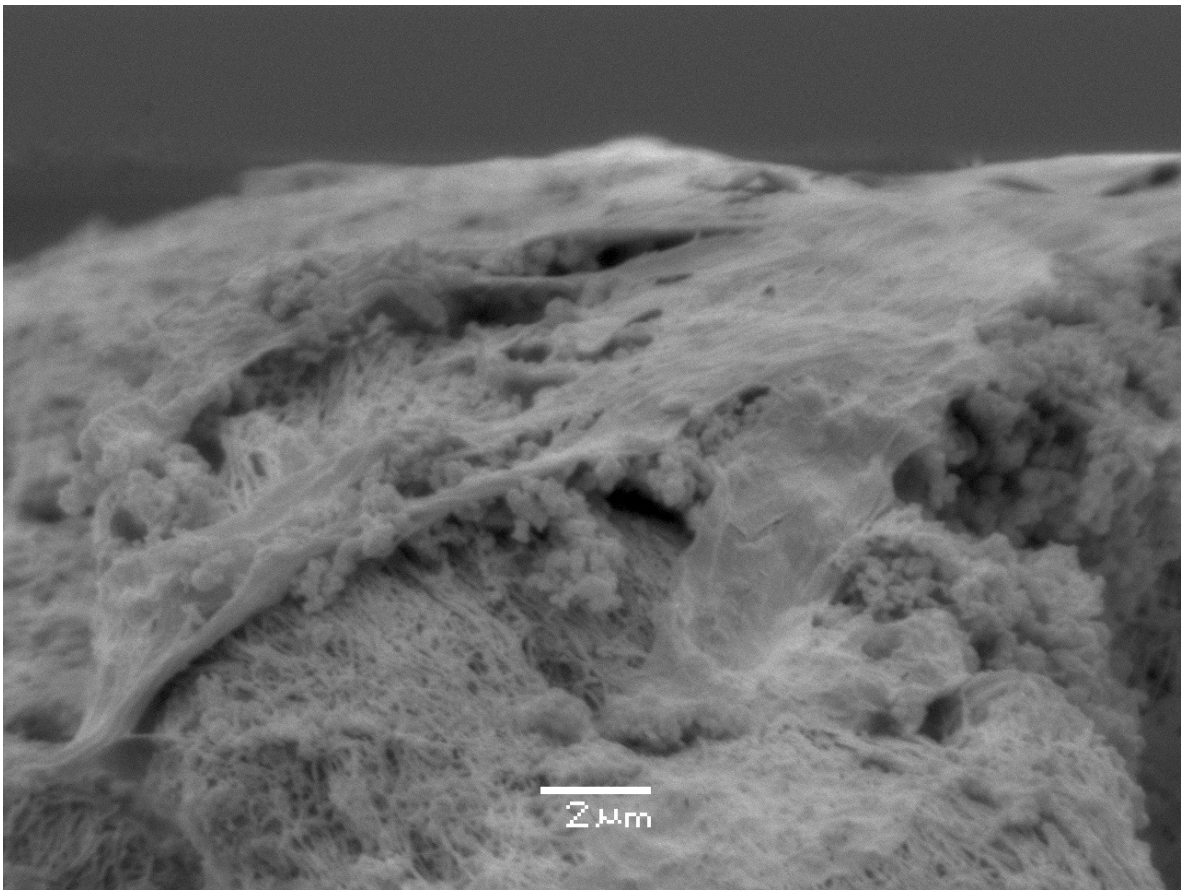


Figure 4.3 Identifies large micro-colonies of predominantly coccoid cells covered in a thin film of EPS denoted with a red arrow (biofilm score 4).

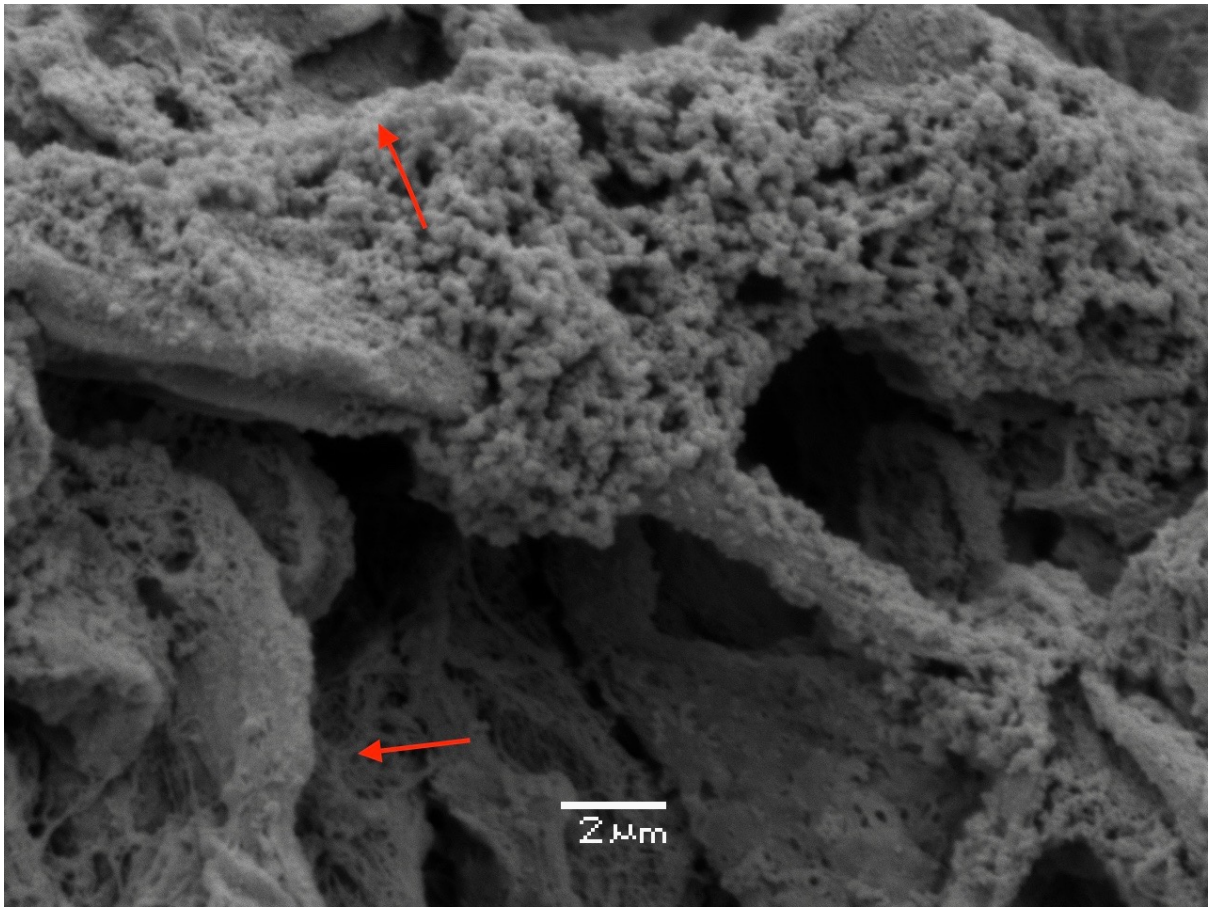
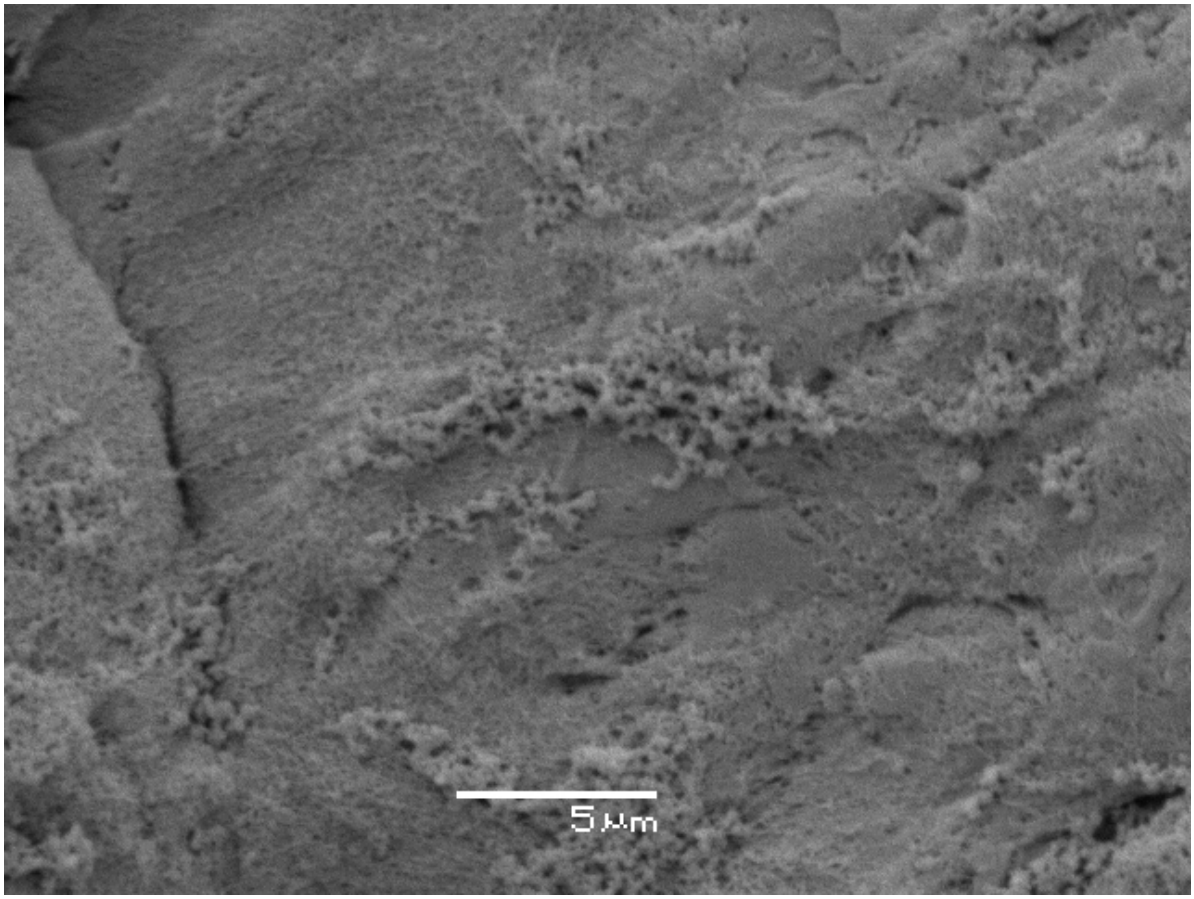


Figure 4.4 Identifies large micro-colonies but less EPS (biofilm score 3).



The spatial organisation of microorganisms was explored using PNA-FISH techniques and identified dense microbial aggregates (biofilm) (Figure 4.5, 4.6). Generally, biofilms were not present in a uniform manner across the entire wound bed. Sampled tissue sections with species-specific and universal bacterial probes, revealed areas of biofilm that were solely mono-species (Figure 4.7) or multi-species biofilms (Figure 4.8). We also identified areas of combinations where both mono-species and multi-species were located within the same sampled tissue section (Figure 4.9).

Figure 4.5 CLSM of biofilm demonstrated via FISH and PNA-FISH. (a) Patient 20, FISH with CLSM shows predominantly Gram-negative rods in biofilm using green-fluro-488-labeled probe targeting *Pseudomonas* spp., and yellow-Cy3-labeled universal bacterial probe.

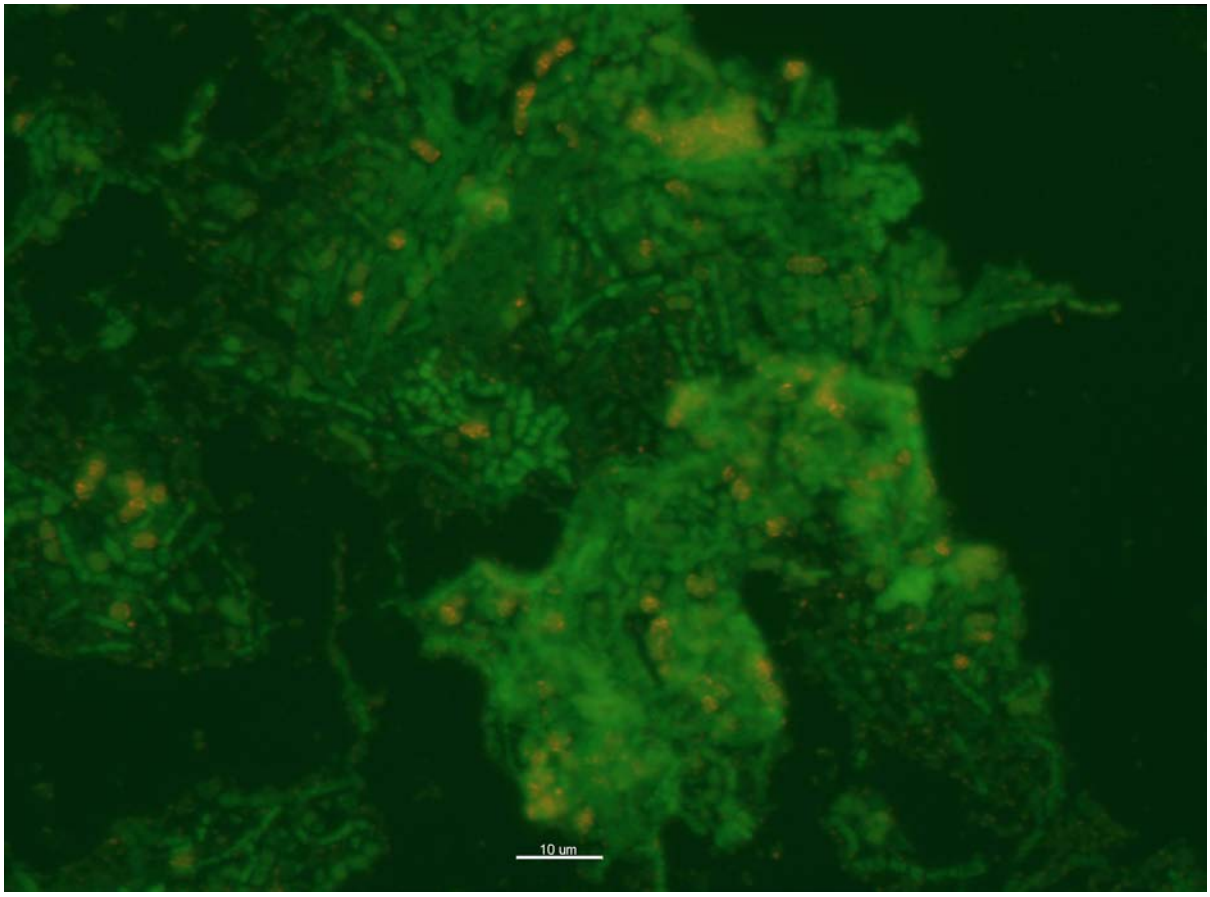


Figure 4.6 Patient 4, PNA-FISH with CLSM illustrates different bacterial morphologies of a multi-species biofilm using fluorescein-labelled universal PNA probes.

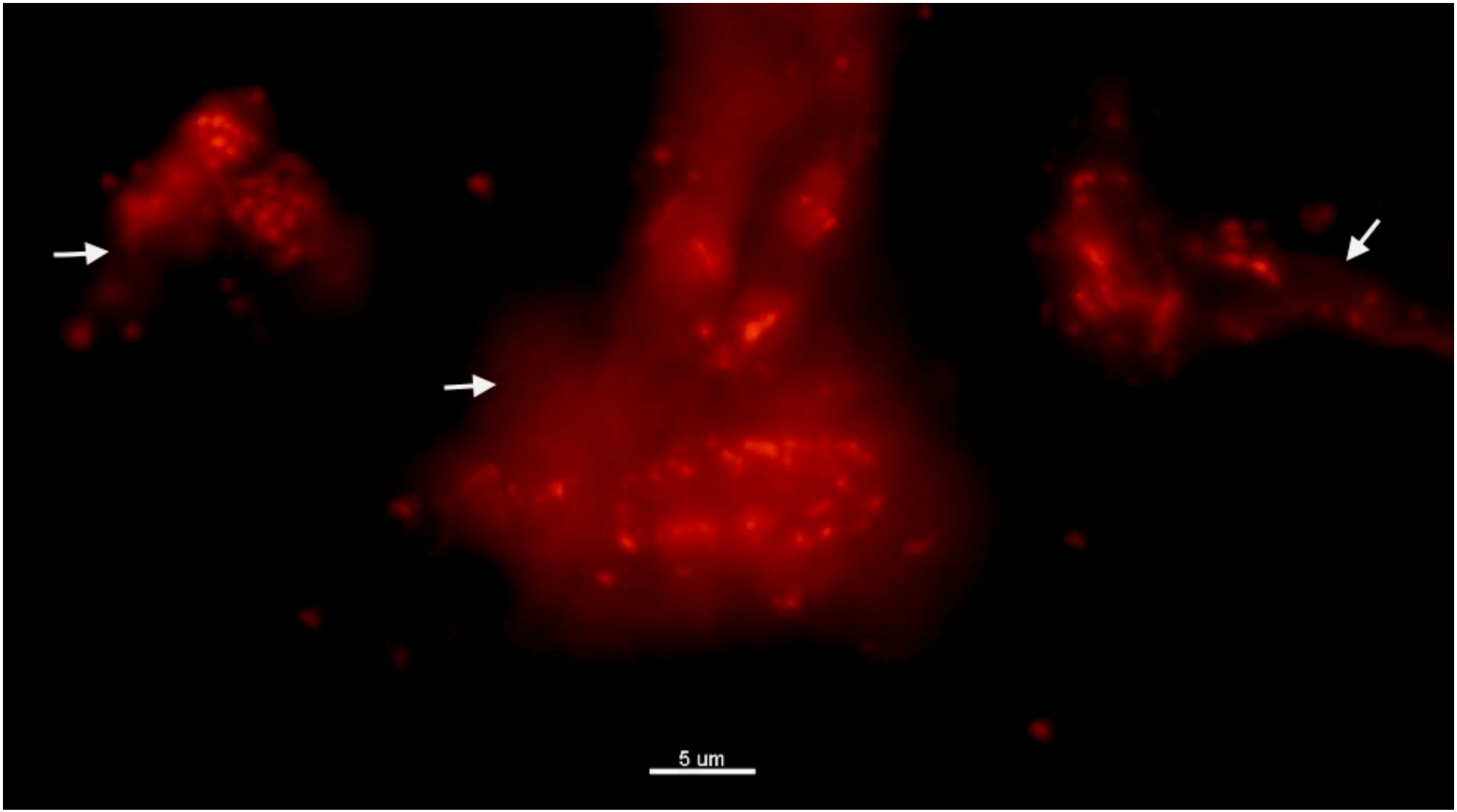


Figure 4.7 FISH and PNA-FISH with CLSM technique to explore the spatial organization of microbial aggregates in DFU samples. Patient 18, identifies two mono-species biofilms in the same wound, bacteria labelled with *S. aureus* PNA probe (green bacteria) and bacteria labelled with coagulase-negative *Staphylococcus* (red).

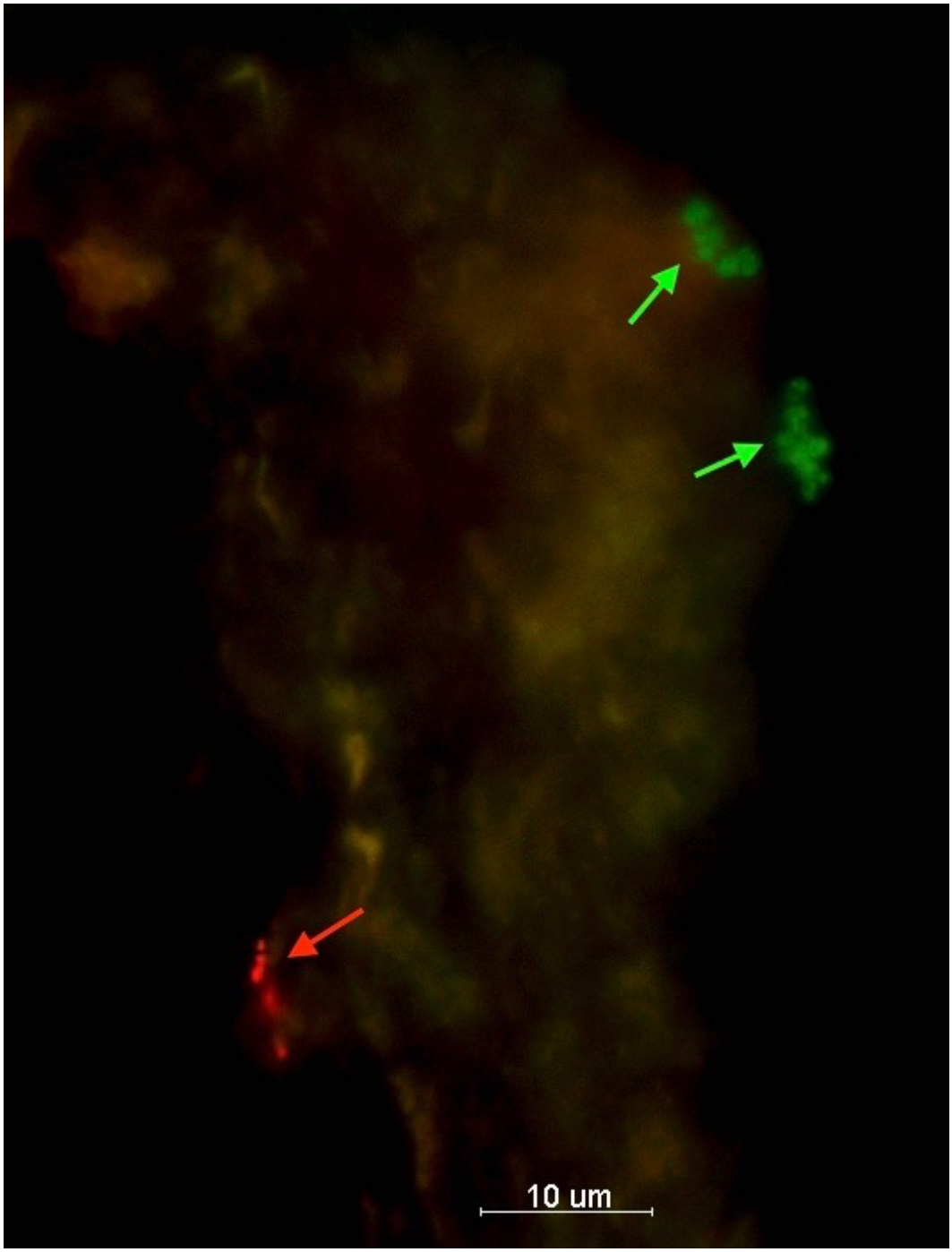


Figure 4.8 Three-dimensional view depicted using the Imaris software of a DFU biopsy. This highlights the structural complexity of biofilms where multi-species biofilm co-exists with mono-species biofilms and planktonic microorganisms. (A) Red-labelled universal PNA probe with (B) *S. aureus* green labelled PNA probe. (C) Mono-species of *S. aureus* biofilm. (D) Planktonic aggregates. (E) Planktonic aggregates of *S. aureus*. Top right-hand corner viewing bubble demonstrates standard CLSM view of multispecies biofilm under high magnification.

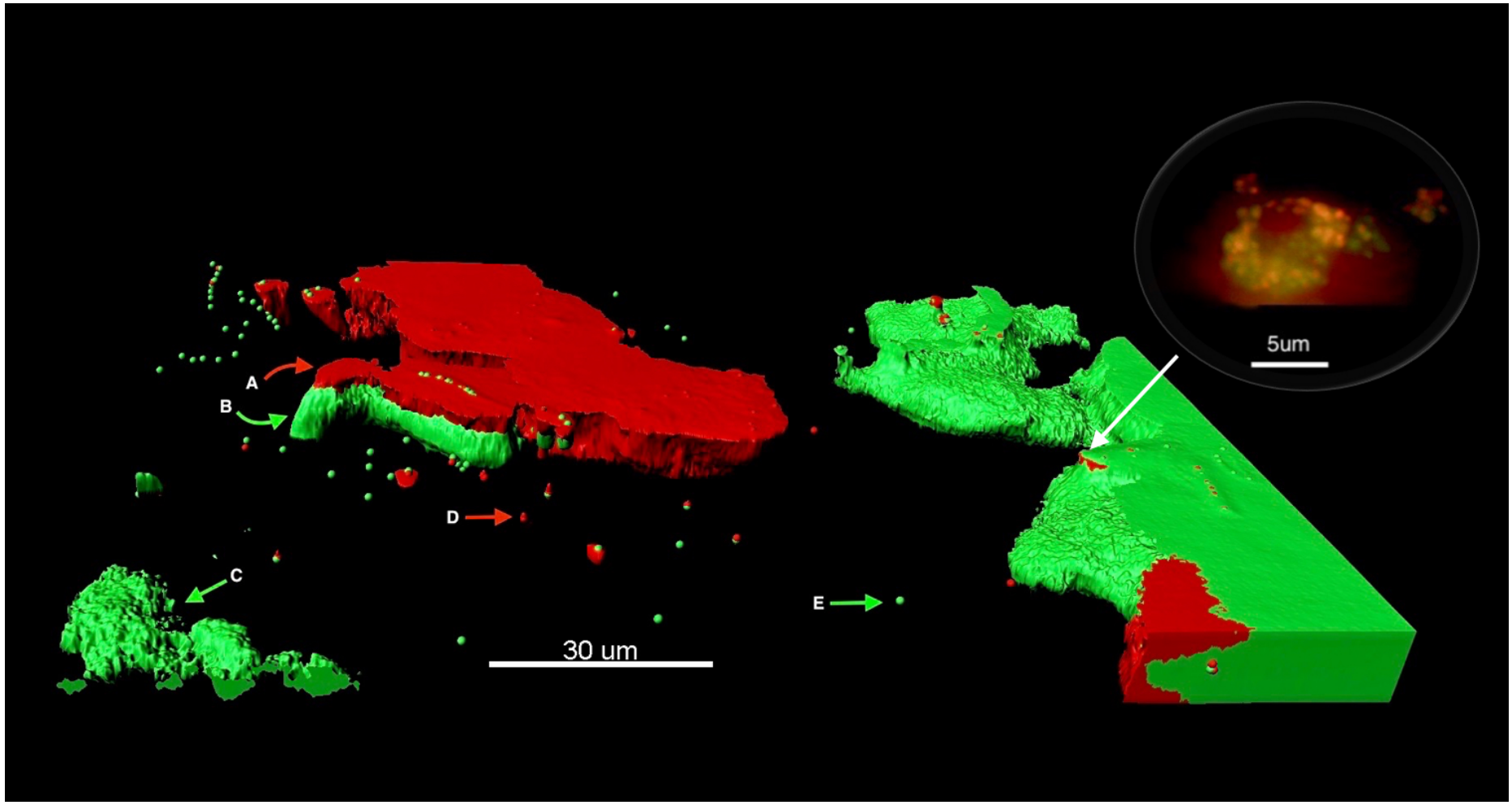
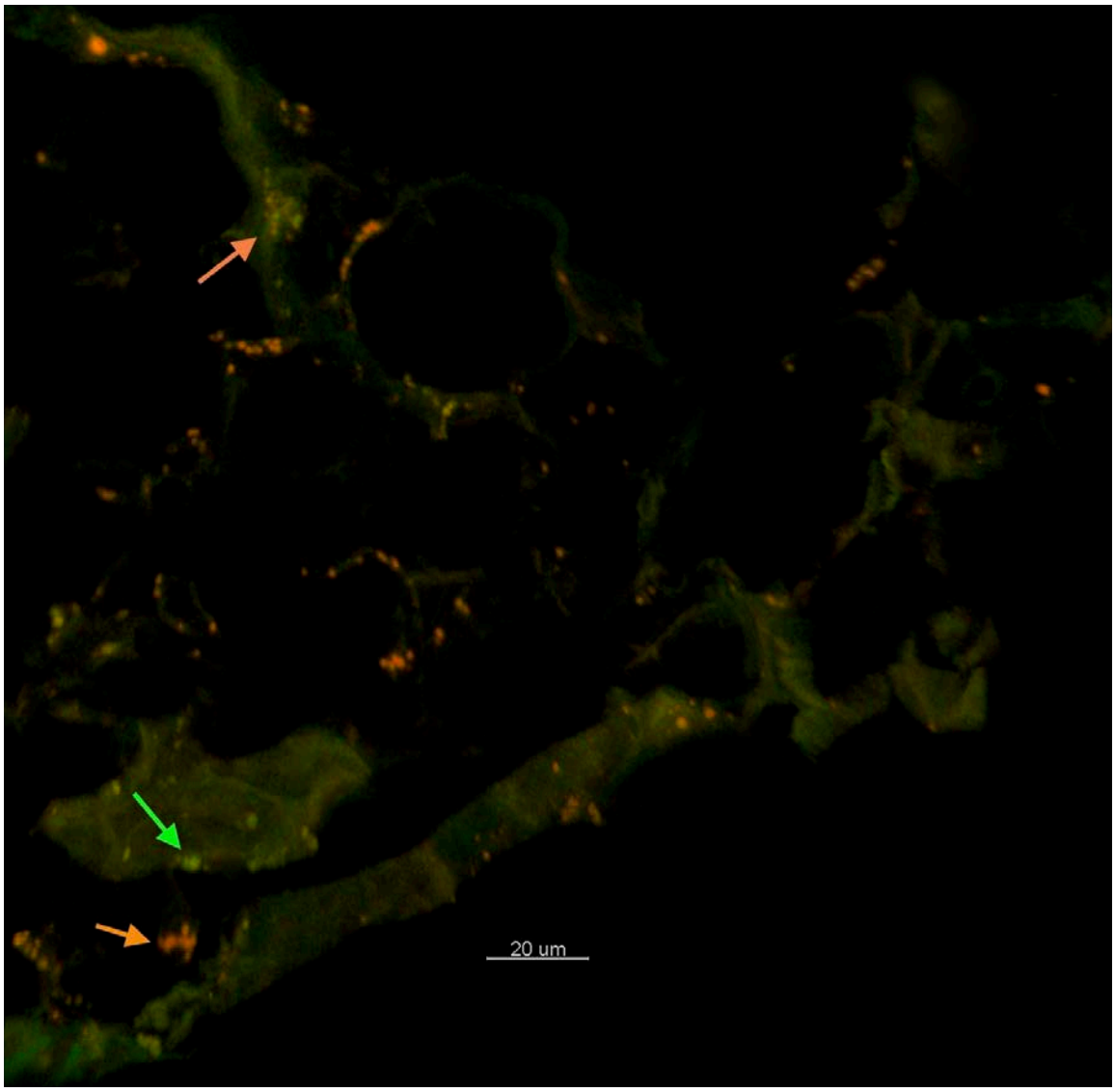


Figure 4.9 Patient 35, FISH with CLSM. Green arrow shows mono-species biofilm (*Staphylococcus* spp. specific probe) and orange arrow shows mixed-species biofilms (universal bacterial red probe).



Clinical wound observations

Using binomial probabilities, the probability of clinical observations associated with the positive presence of biofilm through microscopy were explored (Table 4.2). Except for excessive exudate, the probability of clinicians accurately identifying biofilm using clinical observations was no better than chance alone.

Table 4.2 The probability a clinical wound observation is related to the presence of biofilm. * indicates *p* value < .05. The binominal probability questions asked here is a yes or no response. Therefore, statistical significance should be denoted by an * if the visual marker is accurate in detecting biofilm. The benchmark was set at 50% occurrence rate for the visual marker to be present. The below results indicate that visual markers (with exception of XS exudate) are no better than chance alone.

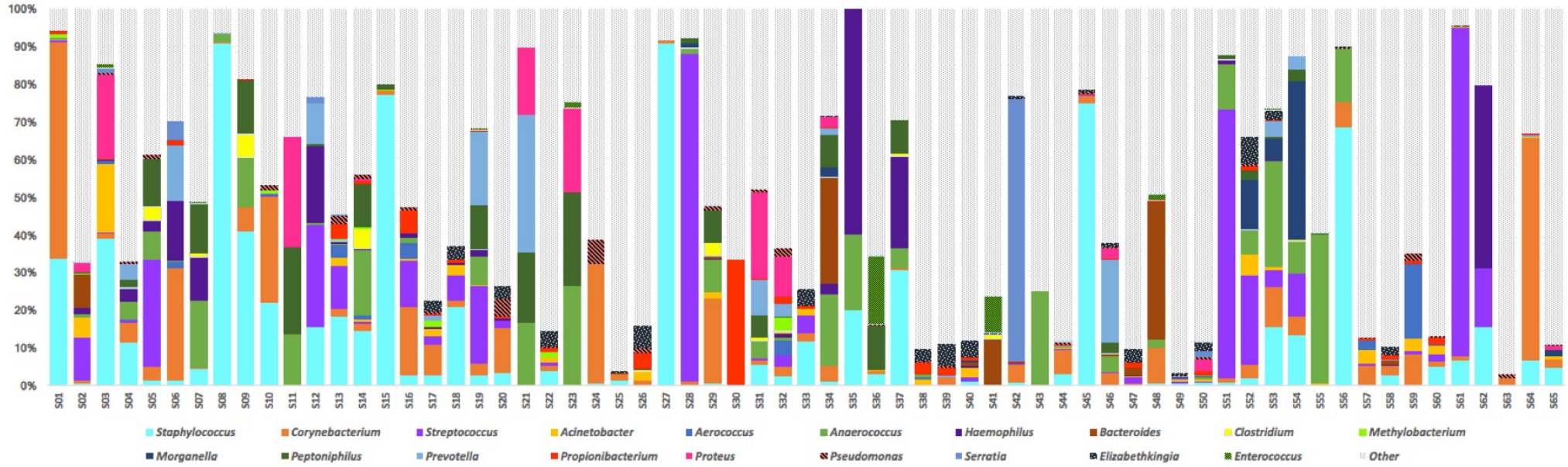
Binomial Test

		Category	N	Observed Prop.	Test Prop.	Exact Sig. (2-tailed)
Presence of slough	Group 1	Yes	38	.58	.50	.215
	Group 2	No	27	.42		
	Total		65	1.00		
XS Exudate	Group 1	No	5	.08	.50	.000*
	Group 2	Yes*	60	.92		
	Total		65	1.00		
Poor tissue quality	Group 1	No	29	.45	.50	.457
	Group 2	Yes	36	.55		
	Total		65	1.00		
Signs of pyocyanin	Group 1	No*	52	.80	.50	.000*
	Group 2	Yes	13	.20		
	Total		65	1.00		
Gelatin Wound Surface	Group 1	No*	46	.71	.50	.001*
	Group 2	Yes	19	.29		
	Total		65	1.00		
Gelatin Reforms Quickly	Group 1	No*	53	.82	.50	.000*
	Group 2	Yes	12	.18		
	Total		65	1.00		

16S rDNA Next generation DNA sequencing

The microbiota of DFUs were explored through 16S rDNA next generation sequencing. Microorganisms contributing greater than 10% relative abundance per individual DFU sample are reported at the genera and species level where possible (Figure 4.10). The most abundant bacteria were (in rank order) *Staphylococcus* spp., *(S. aureus, S. epidermidis), Corynebacterium* spp., *(C. striatum, C. simulans), S. agalactiae, Anaerococcus* spp., *(P. anaerobius), Peptoniphilus* spp,, *Pseudomonas* spp., *(P. aeruginosa) and Prevotella* spp. *(P. melaninogenica, P. nanceiensis).*

Figure 4.10 Bar graph depicting the most common genera of microorganisms in DFUs. The vertical axis refers to relative abundance across DFUs. Horizontal axis is the participant number.



4.1.4. DISCUSSION

Employing a suite of microscopy and molecular approaches to analyse DFU tissue specimens, we identify the presence of densely aggregated colonies (both mono and multi-species) of bacteria often surrounded by an extracellular matrix in tissue biopsies from 65 DFUs. This represents the largest data set in the literature, and supports the view that biofilms are ubiquitous in DFUs and play host to a diverse ecology.

The clinical significance of our findings suggests that biofilms may have a pathogenic role across a spectrum of DFU presentations. We identified biofilm in three different wound states; short duration DFUs (<6 weeks) with acute infection, chronic DFUs with acute infection and chronic DFUs with no infection but non-healing. The visualization of biofilms in chronic non-healing wounds is as expected, where they have been proposed as a likely cause of wound chronicity [141]. The exact mechanisms of biofilm impairment on the healing processes of wounds remain unclear. *In vitro* observations suggest the wound is kept in a vicious inflammatory state preventing the normal wound healing cycles to occur [179,182,184]. Recently, data by James and colleagues (2016) [129] proposed a concept of a localized low oxygen tensions contributing to wound chronicity. Using oxygen microsensors and transcriptomics (examining microbial metabolic activities) to study in situ biofilms, James and colleagues identified steep oxygen gradients and induced oxygen-limitation stress responses from bacteria.

The presence of biofilm however, in wounds of short duration (<6 weeks) presenting with an acute infection is less commonly reported. Five DFUs of short duration were captured in this study (range from 2-5 weeks) with biofilm being visualized in all patients. The general consensus is that biofilms are not responsible for acute infective episodes, for which planktonic

microorganisms are the major driver [115]. People who develop DFUs may be at increased risk of the earlier formation of biofilm. This may be explained by several ill-defined immunological deficits attributed to underlying hyperglycaemia [18], that contributes to a poor response of neutrophils to colonizing or invading microorganisms [340], or from impairments in microbial phagocytosis [341]. Although the number of samples to draw a valid conclusion is small (n = 5), it is unclear whether this phenomenon is specific to DFUs or can be observed in other chronic wound types and presents an interesting trend that warrants further exploration.

In this study, all the presenting infected DFUs had biofilm, but given most DFUs were chronic at presentation we would expect biofilm to be present, but not necessarily involved as an acute pathogen of infection. It is plausible that the biofilm acts a reservoir for bacterial pathogens and biofilm dispersal increases the presence of pathogenic planktonic microorganisms [342]. To support this idea, the most abundant bacteria identified using DNA sequencing in this study was *S. aureus*.

Species-specific probes for *S. aureus* used in our PNA-FISH analysis also confirmed *S. aureus* as being present in the clear majority of samples as dense microbial aggregates. In the absence of direct biofilm culture assays from our clinical isolates, we could refer to the plethora of evidence for *S. aureus* profound ability to form biofilm, particularly on human skin and tissue [343]. Furthermore, *S. aureus* has long been cited as the most common pathogen of infection in diabetic foot infections from culture-dependent studies [42,43]. We also identify *S. aureus* as being the predominant microorganism identified through 16S rDNA sequencing and visualized in biofilm through microscopy in this study. It is also possible that the acute infections of chronic DFUs were caused by a new invasion of planktonic bacteria rather than dispersal from biofilm colonies.

When analysing the community structure of DFUs, multi-species communities comprising of both strict anaerobes and aerobic species were identified. Biofilms can form "microniches" [195], with steep oxygen gradients occurring through biofilm or areas of altered pH or nitrate [344]. These studies confirm that distinct micro niches exist at different depths in biofilms and thus make it possible to understand how metabolically diverse microorganisms coexist. While aerobic Gram-positive cocci were predominant through samples, several strict anaerobes were also present in most samples; *Clostridales family XI* members *Anaerococcus* spp., *Peptoniphilu*s spp., and *Finegoldi*a spp. Using culture-independent approaches, this group of fastidious bacteria have been previously reported as colonizing DFUs in greater abundance when compared against laboratory-based culture techniques [75].

The clinical significance of having multi-species biofilms consisting of metabolically diverse microorganisms (i.e. aerobic and anaerobic microorganisms) is not clear in wound tissue, and there is no direct evidence (from any aspect of human health and disease) to suggest patients with multi-species biofilms have less favourable outcomes then those with mono-species biofilms. Previous reports in the literature however, have identified the occurrence of synergism between metabolically diverse microorganisms that demonstrate a greater pathogenicity / virulence and or an enhanced tolerance to therapeutics [345]. As with most chronic wounds complicated by biofilm, their tolerance to many forms of treatments that include systemic antimicrobials, topical antiseptics and disinfectants is well-documented [172,293]. This has led to expert groups promoting multi-faceted biofilm based woundcare approaches [93] to tackle these tolerant phenotypes.

Part of this biofilm-based approach is the use of systemic antimicrobials in the presence of clinical infection. A question therefore, that needs to be explored is whether clinicians need to

consider altering systemic antimicrobial therapy based on the presence of a multispecies biofilms containing additional "hidden" anaerobes? Most clinicians with access to local and international antimicrobial stewardship guidelines and guidelines specifically for diabetic foot infection, are guided to use empiric first line antimicrobials that provide a broad spectrum of activity against anaerobes (such as Amoxicillin and Clavulanic acid). These guidelines also promote the use of antimicrobials with further targeted anaerobe action (such a Metronidazole). In this instance (and except for biofilm tolerance to antibiotics) most anaerobes are likely targeted by conventional regimens.

One assumption when exploring the microbiota of chronic wounds complicated by biofilm using 16S rDNA sequence techniques, is that the polymicrobial nature of these wounds must in turn equal multi-species biofilms [9]. This is not the case. We identify cases where *S. aureus* forms a mono-species biofilm in one section of tissue which could be located next to a neighbouring multi-species biofilm. This suggests a non-random distribution of microbial biofilms where mono-species biofilm could form in multi-species infections. We do note however, that whilst we identify varying sections of tissue with either mono or polyspecies biofilms, we did not find entire tissue sections containing only mono-species.

This scenario has been previously documented by Bjarnsholt and colleagues (2009) [41] using PNA-FISH on chronic wound samples. They reported that many microbial aggregates were mono-microbial and belonged to either *Pseudomonas aeruginosa* or *S. aureus* (identified using specific probes). Additionally, the depth and location of these microbial aggregates was correlated to the depth of the wound bed. *S. aureus* were primarily located close to the wound surface whereas *P. aeruginosa* was primarily located deeper in the wound bed. The authors concluded that microbial aggregates function in a non-random distribution.

In a recent study on visualizing wound biofilms, the clinical observation of a gel-like substance/film was then correlated to biofilm presence through microscopy [346]. The study concluded that ten of the sixteen samples revealed recurring wound bed film and that this sign was indicative of macroscopic biofilm presence. In contrast, for twenty-six samples analysed in this study, except for excessive exudate, the probability of a clinician accurately identifying biofilm using clinical observations is not better than chance alone.

Furthermore, wounds that exhibit a gel-like substance where biofilm is confirmed through microscopy might have this only in the presence of specific biofilm species as not all wounds exhibit this feature (an example of this could be the mucoid *P. aeruginosa* which produces the viscous polysaccharide alginate in cystic fibrosis). We propose that clinical cues are not useful for detecting biofilm presence in DFUs, regardless of this, our data show that virtually all infected or chronic and non-healing DFUs have biofilm and thus clinicians could just "assume" biofilm will be present. Larger sample sizes from both DFUs and other chronic wound aetiologies are required to verify our results.

In conclusion, microscopy visualization when combined with molecular approaches, confirms biofilms are ubiquitous in DFUs and a paradigm shift of managing these complicated wounds needs to consider anti-biofilm strategies.

CHAPTER 5

5.1 THE EFFECT OF CADEXOMER IODINE ON THE MICROBIAL LOAD AND DIVERSITY OF CHRONIC NON-HEALING DIABETIC FOOT ULCERS COMPLICATED BY BIOFILM *IN VIVO*.

(APPENDIX 5 & 6) STUDY PROTOCOL AND ETHICS APPROVAL

(APPENDIX 9) **MALONE, M.,** JOHANI, K., JENSEN, S. O., GOSBELL, I. B., DICKSON, H. G., MCLENNAN, S., HU, H., VICKERY K. EFFECT OF CADEXOMER IODINE ON THE MICROBIAL LOAD AND DIVERSITY OF CHRONIC NON-HEALING DIABETIC FOOT ULCERS COMPLICATED BY BIOFILM *IN VIVO*. *J ANTIMICROB CHEMOTHER* 2017; 72 (7): 2093-2101. DOI: 10.1093/JAC/DKX099.

Candidate contributions:

- ✓ Worked as primary author for publication
- ✓ Designed the study protocol and methodology
- ✓ Performed all aspects of clinical study, including screening, recruitment, data collection, tissue biopsy sampling and storage.
- ✓ Used genomic software to quality control and analyse DNA sequences from raw files.
- ✓ Used biostatistical approaches to analyse genomic data
- ✓ Used statistical software and knowledge of statistical approaches to analyse / correlate clinical data.
- ✓ Wrote the manuscript in full using information from thesis chapters 1 and 2
- ✓ Reviewed and amended all required changes from co-author contributions
- ✓ Submitted the manuscript as primary author

Abstract

Objectives: The performance of Cadexomer Iodine was determined against microbial populations from chronic non-healing diabetic foot ulcers (DFUs) complicated by biofilm *in vivo*, using molecular, microscopy and zymography methods.

Method: Chronic non-healing DFUs due to suspected biofilm involvement were eligible for enrolment. DNA sequencing and real-time qPCR was used to determine the microbial load and diversity of tissue punch biopsies obtained pre-and post-treatment. Scanning electron microscopy (SEM) and/or fluorescent in situ hybridisation (FISH) confirmed the presence or absence of biofilm. Zymography depicted levels of wound proteases.

Results: 17 participants were recruited over a six-month period. SEM and or FISH confirmed the presence of biofilm in all samples. 11 participants exhibited Log10 reductions in microbial load after treatment (range 1 Log10 - 2 Log10) in comparison to six patients who experienced <1 log10 reduction (p = .04). Samples were tested for levels of wound proteases pre-and post-treatment. Reductions in the microbial load correlated to reductions in wound proteases pre-and post-treatment (p =.03).

Conclusion: To the best of our knowledge, this study represents the first *in vivo* evidence employing a range of molecular and microscopy techniques which demonstrates the ability of Cadexomer Iodine to reduce the microbial load of chronic non-healing DFUs complicated by biofilm. Further analysis correlating log reductions to optimal duration of therapy and improvements in clinical parameters of wound healing in a larger cohort are required.

5.1.1. INTRODUCTION

In a person with diabetes, foot ulceration leaves a physical break in the protective barrier of the skin. Factors including a retarded host immune response and pathogen-related dynamics (such as virulence or pathogenicity) may predispose the DFU to further planktonic microbial replication and invasion. This may result in damage to host tissues and an inflammatory response that is characterized as a clinical infection [2].

Increasing evidence into the role of microorganisms involved in DFUs (and other wound aetiologies) have identified single free-floating microorganisms (planktonic) that are responsible for acute infections, may not necessarily represent the ecology of microorganisms present in chronic non-healing wounds. Instead, the focus has orientated towards the concept of biofilms which differ markedly in both their physiology and activity. The exact mechanisms of biofilm impairment on the healing processes of wounds are not clear, but general consensus suggests that biofilms maintain an elevated inflammatory state within the wound.[144]

Microorganisms in biofilm exhibit enhanced tolerances to chemical, biological and host attack than planktonic forms. *In vitro* biofilm models have demonstrated that microbial biofilms can withstand antimicrobial concentrations 100 to 1000 times higher than that of planktonic counterparts [145]. This may go towards explaining why some wounds fail to heal with standard care and why chronic infections persist [141]. *In vitro* models assessing the effectiveness of many antimicrobials used in wound related products have identified these treatments often have variable and poor results against microbial cells in biofilm phenotype [294,297,347].

One potential explanation for this treatment failure is because the wound care treatments do not target or are ineffective against biofilm. Cadexomer Iodine however, has demonstrated superior efficacy against microbial biofilms when tested *in vitro* and in animal models, when compared against other topical antimicrobials used in wound-care dressings [294,297,347].

Therefore, a pilot study using 16S rDNA next generation sequencing, real-time qPCR, microscopy techniques (SEM and FISH) and gel zymography, was used to identify if the topical antimicrobial Cadeoxmer Iodine could reduce the microbial load of chronic non-healing DFUs complicated by biofilm *in vivo.* Additional interests were to explore the effects of Cadexomer Iodine on the microbial communities pre-and post-treatment and determine the levels of wound proteases (MMP-2 and MMP-9). It was not the intention of this pilot study to correlate any effects of microbial loads or diversity to wound healing outcomes.

5.1.2. MATERIALS AND METHODS

Ethics

Ethics approval for this study was granted by the South West Sydney Local Health District Research and Ethics Committee (HREC/14/LPOOL/487, SSA/14/LPOOL/489).

Exclusion criteria

Patients with clinical signs of infection as per the Infectious Disease America guidelines [2], known osteomyelitis that was associated with the DFU, or patients who had received any topical or systemic antimicrobial therapy two weeks prior to enrolment, were not eligible. The reason for excluding patients with clinical infection was the assumption that these wounds would be driven predominantly by planktonic microorganisms [348]. Participants with general

contraindications to the use of Cadexomer Iodine as per the manufacturers guidelines were also excluded [349].

Subjects and sample collection

Individuals were eligible for the study if they had a chronic non-healing DFU defined as a wound greater than six-weeks duration failing to respond to standard care [350]. Cadexomer Iodine was applied every second day over a seven-day treatment period (total of 3 applications). Sharp debridement of DFUs were withheld over the seven-day treatment period given this was likely to significantly impact results [309]. A tissue biopsy was obtained from the wound edge for each participant before and after treatment. All tissue samples were frozen at -80°C until completion of the last patient and processed in bulk as to reduce any bias. Additionally, we collected broad demographics and wound metrics. Primary endpoint was a reduction in microbial load seven days post-treatment. Secondary analysis included the exploration of community richness and diversity of DFUs pre-and post-treatment, visual changes to biofilm structures and alteration to levels of wound proteases (MMP-2 and MMP-9).

Cadexomer Iodine (Iodosorb®, Smith and Nephew)

Iodosorb ointment is designed as a carrier system enabling the delivery of Iodine which can penetrate the cell wall of microorganisms and disrupt protein and nucleic acid structure and synthesis [351]. Iodosorb® consists of small polysaccharide beads (Cadexomer) containing 0.9% iodine, which, in the presence of wound exudate causes the polysaccharide beads to swell allowing a slow sustained release of Iodine into the wound.

Specimen collection and storage

A 3mm (width) x 10mm (depth) tissue punch biopsy was obtained from the edge of each DFU after cleansing the wound with NaCl 0.9%. Tissue biopsy samples were obtained for all participants at baseline and day seven. For SEM, an additional 2mm (width) x 10mm (depth) was necessary. Following removal, tissue samples were rinsed vigorously in a phosphate buffer solution (PBS) bath to remove any coagulated blood and to reduce the number of planktonic microorganisms. Samples were cut transversely into two 1.5mm pieces for DNA sequencing and FISH. DNA samples were immediately placed into RNA*later*® (Ambion, Inc) for 24 hours at 4°C and then frozen at -80°C until DNA extraction. Tissue samples for FISH were immediately fixed in 4% paraformaldehyde overnight at 4°C, then transferred into PBS and frozen at -80°C. Tissue samples for SEM were immediately fixed in 3% glutaraldehyde overnight at 4°C, then transferred into 0.1% phosphate buffer (PB) and frozen at -80°C. All tissue samples remained at -80°C until study completion to reduce any bias and were processed in bulk.

Tissue processing workflow

DNA Extraction

Tissue samples were defrosted on ice prior to DNA extraction and tissue samples were weighed individually (range of weights; 5 – 10 mg of human tissue). Genomic DNA was extracted using Mo Bio PowerBiofilm DNA isolation kit (Mo Bio Cat# 24000-50) following the manufacturer's instructions.

16S rDNA next generation sequencing to determine bacterial diversity

16S rDNA sequencing was carried out by a commercial laboratory (Australian Centre for Ecogenomics) targeting the V4 region of the 16S rDNA using eubacterial universal primers

515F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
CCAGCAGCYGCGGTAAN) and 806R
(GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
GGACTACHVGGGTWTCTAAT).

Preparation of the 16S rDNA library was performed as described at the Australian Centre for Ecogenomics using the workflow outlined by Illumina (San Diego, California, United States). In the 1st stage, PCR products of ~466bp were amplified according to the specified workflow with an alteration in polymerase used to substitute Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, Massachusetts, United States) in standard PCR conditions. Resulting PCR amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, California, United states). Purified DNA was indexed with unique 8bp barcodes using the Illumina Nextera XT 384 sample Index Kit A-D (Illumina FC-131-1002) in standard PCR conditions with Q5 Hot Start High-Fidelity 2X Master Mix. Indexed amplicons were pooled together in equimolar concentrations and sequenced on MiSeq Sequencing System (Illumina) using paired end sequencing with V3 300bp chemistry.

Sequence analysis and quality control

Reads in FASTQ format were imported to CLC genomics workbench version 8.5.1 using the microbial genome finishing module (CLC bio, Qiagen Aarhus, Denmark), for sequence quality control and analysis. Workflows for sequence quality control and operational taxonomic units (OTU) clustering were based on previously reported wound microbiota analysis [10]. OTUs were defined as molecular proxies for describing organisms based on their phylogenetic relationships to other organisms and were reported at either the genera or species level identification where possible.

Briefly, after sequence and quality control measures, reads were assigned to OTUs using SILVA [316] at 97% similarity at the genus level and species level where possible. OTUs were aligned using MUSCLE [10] to reconstruct a phylogenetic tree, allowing the estimation of the alpha diversity pre and post treatment for each DFU. This included both community richness (Rarefaction) and community diversity (Shannon Weaver Index). Rarefaction curves allow the estimation of the number of unique microbial taxa within a sample and the Shannon Weaver Index is a measure of diversity that includes the number of unique microbial taxa and their relative evenness within each sample. Thus, a higher Shannon Weaver Index correlates to a greater diversity.

qPCR to determine microbial load in DFU biofilms

We utilised real-time quantitative PCR (qPCR) to determine microbial load in DFU biofilms as previously reported [322,323]. To quantify the total amount of bacterial DNA per mg of tissue in each wound tissue sample we obtained the copy number of the 16S rDNA (copies/µl), which was normalised against the human 18s rDNA (copies/µl) in each chronic wound sample.

Characterization and visualization of DFU biofilm

The presence or absence of biofilms in chronic non-healing DFUs, was confirmed through SEM or FISH. For this study, we used definitions promoted by an expert group [2] to characterise biofilm as being; microbial aggregates surrounded by a self-produced or host derived matrix adhering to natural or artificial surfaces in the host, or aggregates associated with but not directly adherent to the surface [93].

Fluorescent in situ hybridisation

2-3 millimetres of DFU tissue were embedded in optimal cutting temperature (OCT) embedding matrix (Tissue-Plus™ O.C.T Compound, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and frozen at –80°C overnight. DFU tissue were sectioned to a thickness of 6 μm and mounted on SuperFrost Plus slides (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For visualisation of microorganisms and biofilm, confocal laser scanning microscopy were combined with fluorescence in situ hybridization. The Hybridisation process used was previously described by Thurnheer *et al* (2004) [335].

Briefly, two different probes were utilised for in situ hybridisation; a) Fluro 488 labelled universal probe [337] (final concentration 5 ng/ µl) Cy3 labelled *Staphylococcus spp*. specific probe (final concentration 5 ng/ µl) [352]. All images were examined under confocal laser scanning microscope (Carl Zeiss Ltd., Herefordshire, UK). All images were processed using ZEISS ZEN Imaging Software (black edition).

Scanning Electron Microscopy and image interpretation

In vivo microbial biofilms associated with DFU tissue were sampled at 5–200 µm for optimal visualisation through scanning electron microscopy [120]. 2-3 millimetres of DFU tissue were fixed in 3% glutaraldehyde, followed by 3 washes of 0.1M phosphate buffer prior to serial ethanol dehydration and hexamethyldisilazane (Polysciences, Inc., Warrington, Pa.) as described previously.[323] Dried samples were coated with 20-nm gold film and examined using SEM. Each sample was scored based on the amount of bacteria/biofilm observed using an arbitrary five-point scale as previously reported [78]. Score 0 = no bacteria observed; score 1 = single individual cells; score 2 = small micro-colonies (~ 10 cells); score 3 = large micro-colonies (~100 cells); score 4 = continuous film; score 5 = thick continuous film.

Determination of wound proteases

Wound fluids were collected from diabetic foot ulcers pre and post treatment and stored at -80°C for quantitation of matrix metalloproteinase (MMP-2 and total MMP-9) by gel band zymography as previously described [353].

Statistics

Wound metrics and microbiota data were analysed through Statistical Package for Social Sciences Version 23 (SPSS Inc., Chicago, Illinois, USA). Wilcoxin signed- rank test for paired samples of non-parametric data were performed on pre-and post-treatment microbial Log10 reductions. Chi-square was used to correlate OTUs and Shannon indices to Log10 reduction. One-way ANOVA was used to estimate variances between MMP levels before and after treatment. Logistic regression was employed to correlate microbial reduction to MMP levels. CLC genomics workbench version 8.5.1 in combination with the microbial genome-finishing module (CLC bio, Qiagen Aarhus, Denmark) were used to analyse DNA sequence data. For all comparisons and modelling, the level of significance was set at $p < .05$. Data are given as mean, standard deviation (±) and 95% confidence interval (CI).

5.1.3. RESULTS

A total of 17 patients with chronic non-healing DFUs were enrolled. One ulcer from each patient was biopsied pre-and-post treatment. Patient demographics and wound metrics are shown in Table 5.1.

Table 5.1 Patient demographics and wound metrics at baseline.

Patient demographics	
Male	11 (65%)
Female	6 (35%)
Type 1 Diabetes	2 (12%)
Type 2 Diabetes	15 (88%)
Mean age in years	66 (± 13.6)
Wound Metrics	
Location of ulcer	
Plantar metatarsal head	8
Calcaneum	3
Dorsal foot	2
Ankle	2
Hallux	2
Duration of ulcer in weeks	
Mean duration of ulcer at baseline	25 (± 20.7, range 6 to 72)
University of Texas Wound Classification	
1A	7 (41%)
1C	1 (41%)
2A	2 (12%)
2C	1 (6%)
Size of ulcer	
Mean DFU size at baseline	3.7 x 2.7 cm (L x W)

Microbial load was determined via qPCR for all 17 DFU samples pre-and post-treatment. However, three samples were removed from exploring community richness (Rarefaction) and diversity (Shannon Index) due to a low number of 16S rDNA reads, leaving twelve samples available for analysis. In total (for these twelve samples), 703, 346 high quality DNA sequences were generated (before = 384,772, after = 318,574), with a median of 31,452 per sample level data (Range = 1,137 to 61,820). The clustering of OTUs identified 1,976 unique taxa of which low abundance OTUs were removed (<0.1%), leaving 368 OTUs for further analysis. Only eight wound fluid samples were available for analysis MMPs with the remaining sample protein concentrations too low.

Confirmation of the presence or absence of biofilms in each DFU

The presence of biofilm was visualized and confirmed in all 17 participants using SEM, FISH or both (Figures 5.1, 5.2). Biofilm architecture was graded (via SEM) using an arbitrary sliding scale previously reported [78]. The median value of DFU biofilm architecture reduced between pre and post treatment samples; pre-treatment median was four (large micro-colonies ~100 cells, and a continuous film/matrix) and the post-treatment median was three (large micro-colonies ~100 cells).

Figure 5.1 SEM image from a pre-treatment (Cadexomer iodine) DFU tissue sample. Microbial aggregates with self-produced or host derived matrix that we describe as a stringy like glue appearance. SEM imaged at 5µm

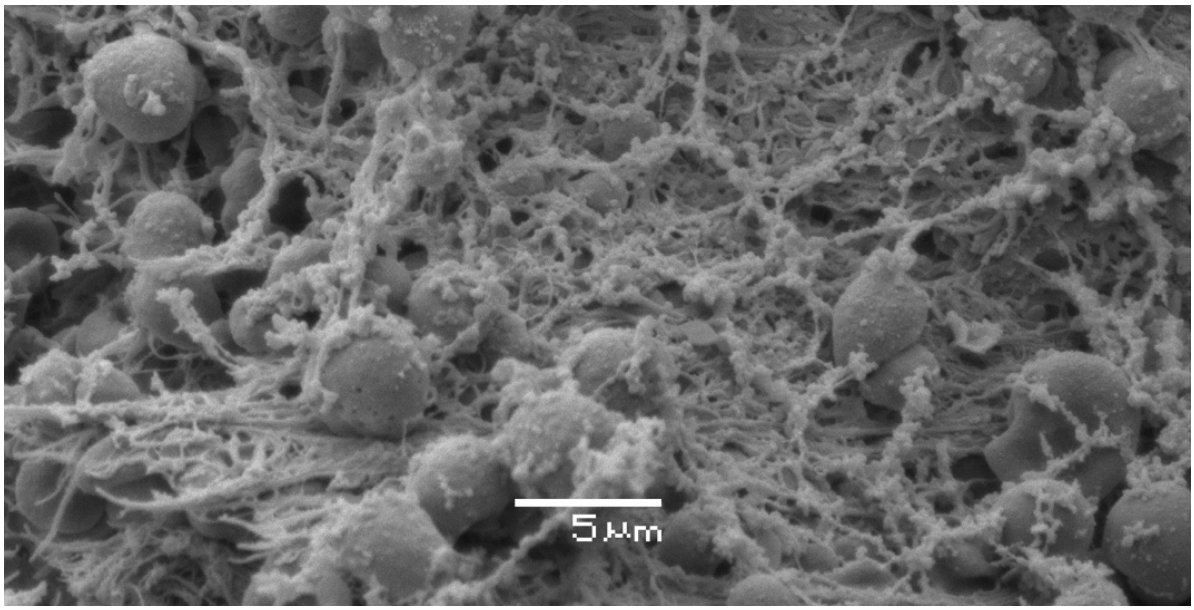
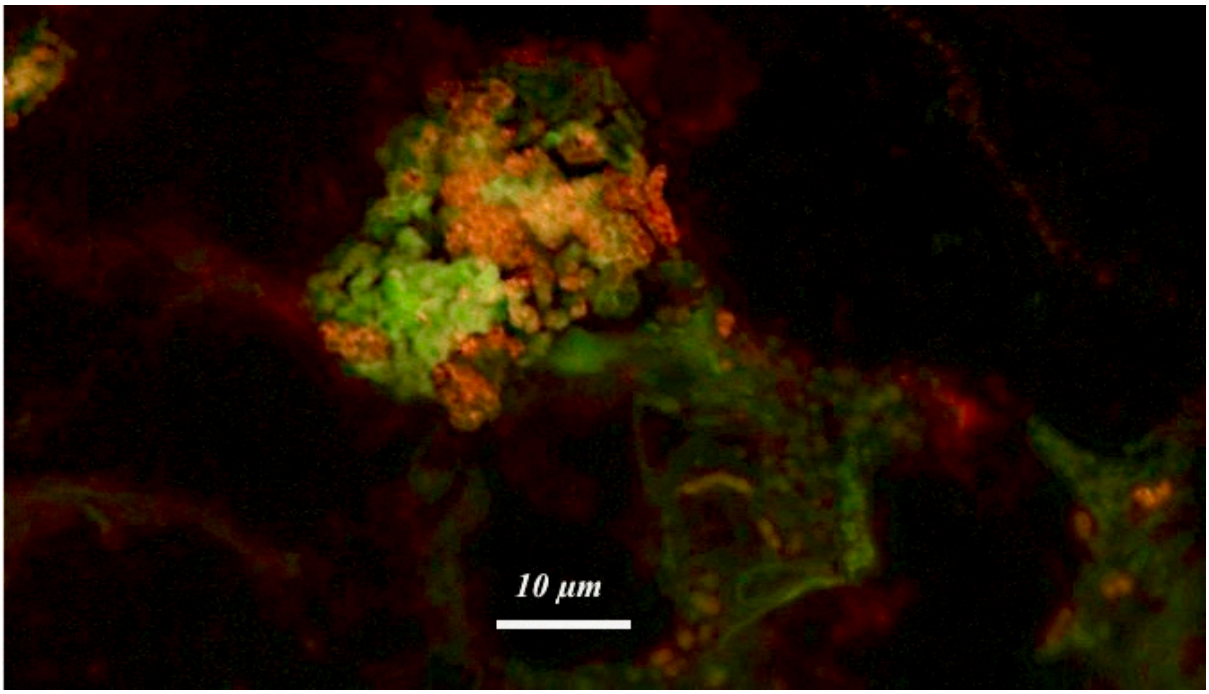


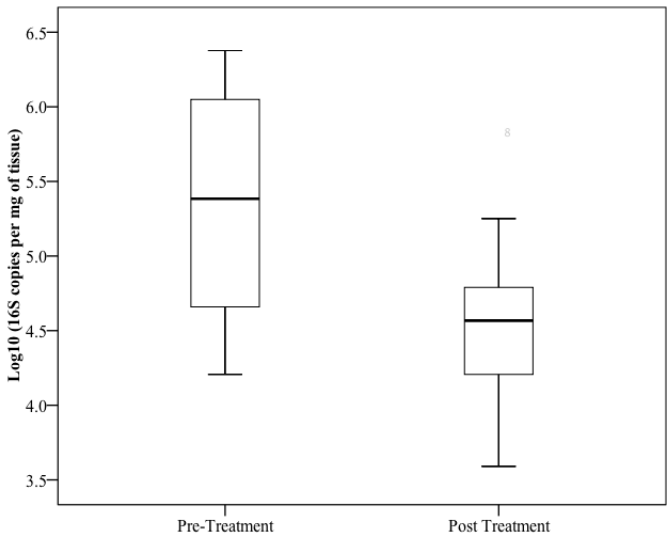
Figure 5.2 FISH image pre-treatment (Cadexomer iodine) of the same DFU tissue sample in figure 5.1. Image depicts microbial aggregates of mixed species biofilm (Fluro 488 labelled universal bacterial probe [red] and Cy3 labelled *Staphylococcus spp.*, specific probe).



Reduction in microbial load of chronic non-healing DFUs complicated by biofilm

The application of Cadexomer Iodine resulted in 11 samples achieving up to and greater than a 1-log10 reduction (mean log10 microbial load pre-treatment = 8.38^{5} 16S copies / per mg of tissue versus 3.69^{4} 16S rDNA copies / per mg of tissue, p = .02, 95% CI 7.21^{4} to 8.37^{5}) (Figure 5.3). Six samples had no change or increases in log10 (mean log10 microbial load pre-treatment = 1.69^{5} 16S rDNA copies / per mg of tissue versus 1.59^{5} 16S rDNA copies / per mg of tissue).

Figure 5.3 Effect of Cadexomer Iodine pre-and post-treatment. Bar graph represents the mean log10 values of all 17 patients.



Analysis of community richness and diversity of chronic non-healing DFUs treated with Cadexomer Iodine.

The most abundant OTUs (based on the number of DNA sequences represented when pooling samples) are noted in Figure 5.4. The richness and diversity of chronic non-healing DFUs pre- and post-treatment are also reported at the individual sample level of microorganisms contributing to >5% of abundance in rank order (Table 5.2). Post-treatment identified diversity shifts with increasing environmental contaminants. These microorganisms are identifiable only by molecular methods (in the majority) and included in Proteobacteria-E01-9C-26 Marine Group, Proteobacteria-ARKDMS-49, Archaea-Cenarchaeum, Elizabethkingia spp., Bacteroidetes-Rhodothermacae and Proteobacteria-Rhodothalssium. Furthermore, some of these microorganisms are known extremophiles, existing in hostile, niche environments.

Figure 5.4 Denotes the most frequent microorganisms based on the pooling of all DNA sequences, and the total number of DNA sequences attributed to each microorganism.This graph also exemplifies the contrast between low and high frequency taxa at the genus/species level. High-frequency taxa consist of a few predominant microorganisms (typically between 1 to 9 microorganisms, samples 31, 32, 36, 42, 44, 45, 91), or lower-frequency taxa of multiple microorganisms (typically ten-fold in comparison to high frequency taxa, >30 to 90 microorganisms, samples 33, 38, 39, 40, 41).

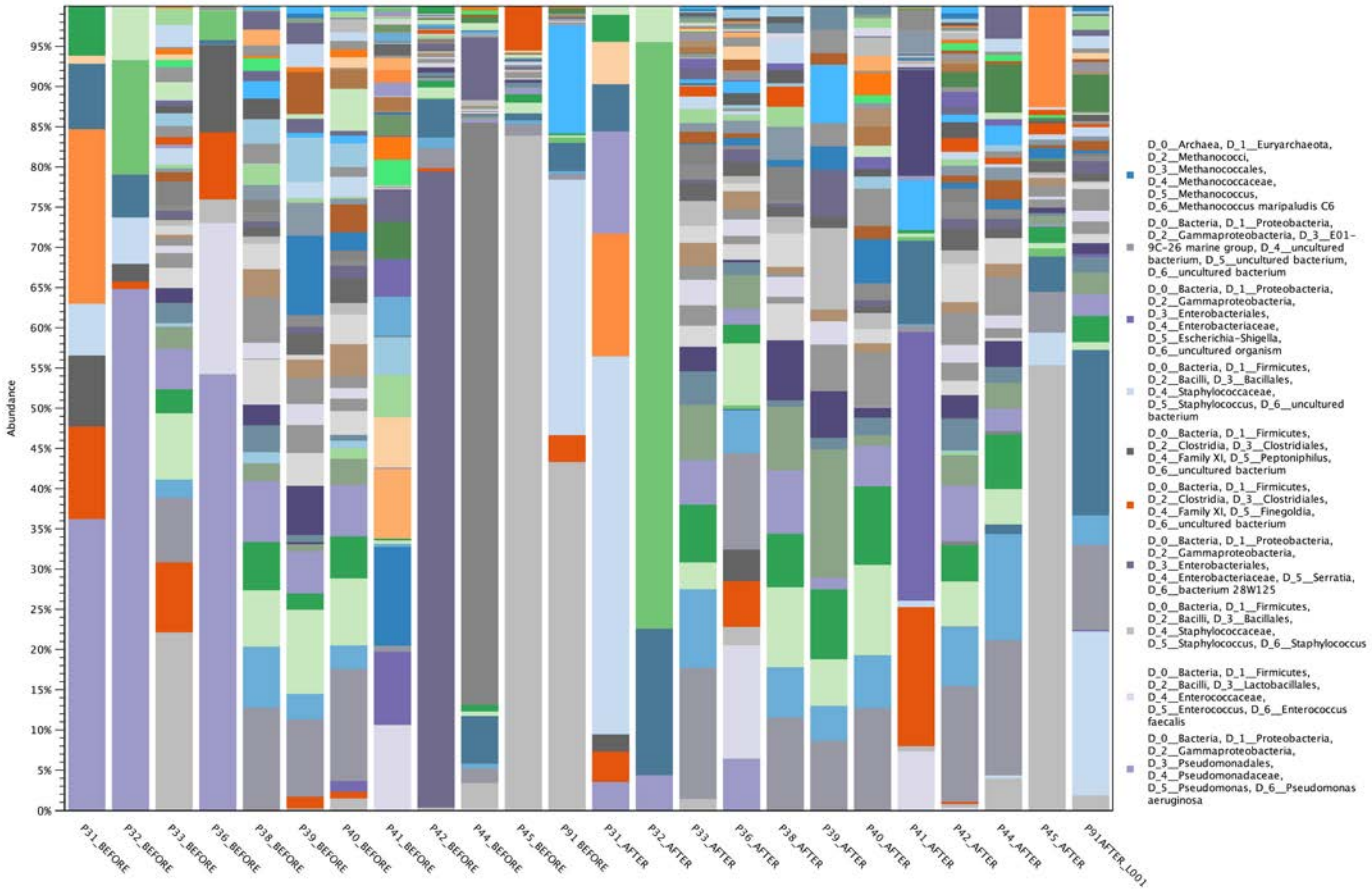


Table 5.2 Pre-and post-treatment community diversity in microorganisms contributing to >5% abundance of each individual sample.

PRE-TREATMENT				**POST-TREATMENT**			
Genera	**No of wounds**	**Avg. abundance (%)**	**Aerotolerance**	**Genera**	**Samples**	**Avg. abundance (%)**	**Aerotolerance**
Pseudomonas spp	5	58.5	Aerobe	*E01-9C-26 Marine*	8	10	Unknown
E01-9C-26 Marine	4	11.2	Unknown	*ARKDMS49*	7	5.7	Unknown
Staphylococcus spp	4	58	Facultative	*Cenarchaeum*	5	5.1	Unknown
Rhodothermaceae spp	3	5	Unknown	*Cyanobacteria-subsectionl*	5	7.4	Unknown
Finegoldia spp	3	7.8	Anaerobe	*Rhodothermaceae* spp	4	5.8	Unknown
Elizabethkingia meningoseptica	3	6.3	Aerobe	*Rhodothalassium* spp	3	7	Unknown
Cornyebacterium spp	3	5.3	Aerobe	*Cornyebacterium* spp	3	11	Aerobe
Peptoniphilus spp	2	9.5	Anaerobe	*Elizabethkingia meningoseptica*	3	6.6	Aerobe
Ananerococcus spp	2	9.2	Anaerobe	*Staphylococcus* spp	3	42.8	Facultative
Proteus penneri	2	20.5	Facultative	*Proteus penneri*	2	39	Facultative

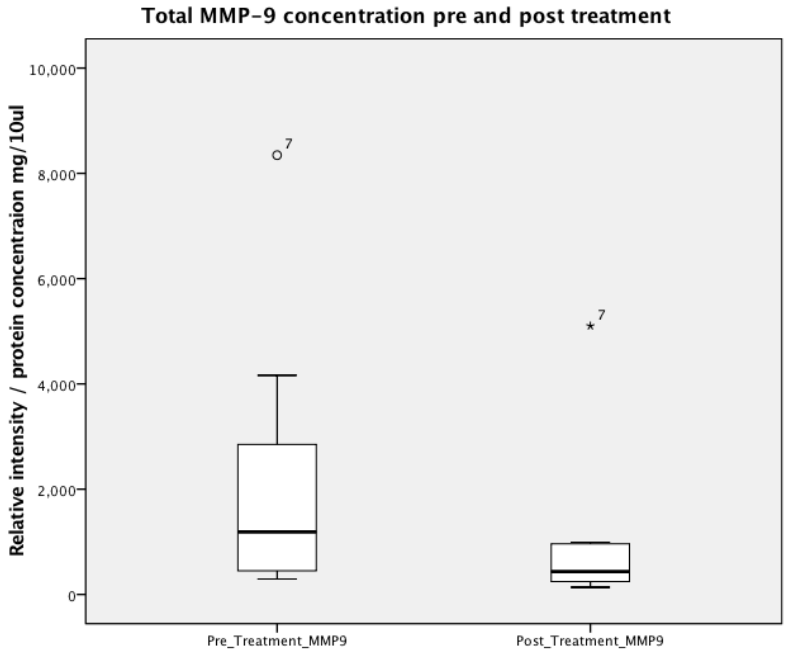
The taxonomic richness and diversity of pre-and post-treatment pooled samples identified no differences between mean OTUs (Pre-treatment = 115, SD 69.3 versus Post-treatment = 112, SD 74.3, p = .53) and mean Shannon indices (Pre-treatment = 2.8, SD 1.2 versus Post-treatment = 3, SD 1.4, p = .58). When exploring this at the individual sample level there was large heterogeneity between pre-and post OTU and Shannon indices. To ascertain if the number of unique OTUs and their relative abundance were correlated to reductions in Log10 values, a chi-square was performed. Reductions in Log10 did not correlate to reductions in either OTUs (p = .85) or Shannon indices (p = .72).

MMP levels pre-and post-treatment

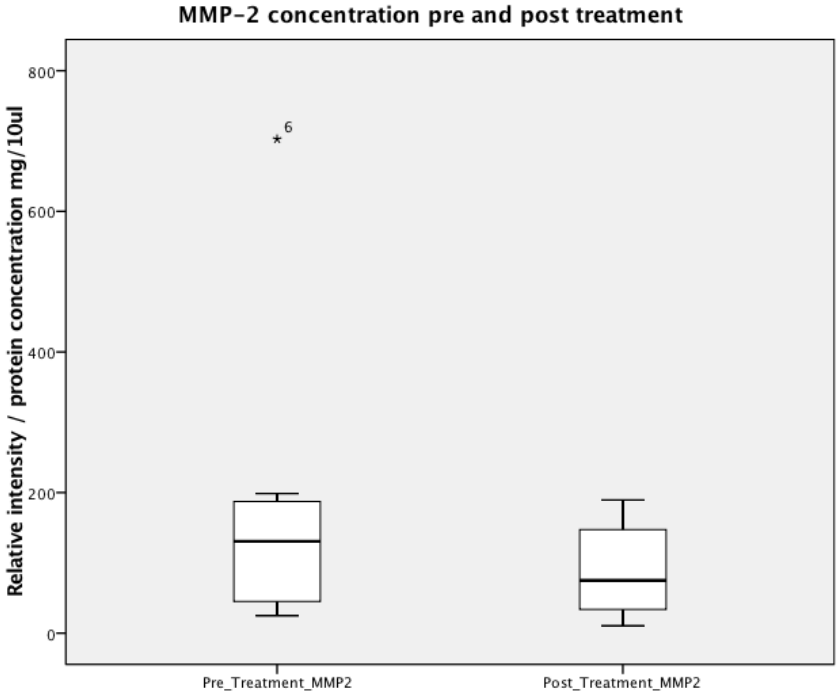
Mean total MMP-9 and MMP-2 levels in eight samples reduced following seven days' application of Cadexomer Iodine (Figure 5.5). However, only total MMP-9 reached statistical significance (mean total MMP-9 before = 2202 mg/10uL versus mean total MMP-9 after = 1065 mg/10uL, 95% CI -14.5 to 2287, p = .05). Mean MMP-2 before =181.9 versus mean MMP-2 after = 89, 95% CI -61.3 to 246.7, p = .197). In general, any reductions in the levels of MMPs were correlated to reductions in the microbial load (p = .03).

Figure 5.5 (a) total MMP-9 values pre-and post-treatment. (b) MMP-2 values pre-and post- treatment.

(a)



(b)



5.1.4. DISCUSSION

Using a combination of DNA sequencing, qPCR, SEM and FISH, the ecology of wounds and presence of microbial biofilms was explored. To the best of our knowledge, we are the first to employ these suites of molecular and microscopy techniques to show that Cadexomer Iodine can reduce the microbial load of chronic non-healing DFUs complicated by biofilm. We also show that in reducing microbial load, concomitant reductions in wound proteases are also achieved.

16S rDNA sequencing allowed the exploration of chronic non-healing DFU microbiota and provided useful insights that these wounds support complex polymicrobial communities. Molecular methods also demonstrated that Cadexomer Iodine had a broad level of antimicrobial activity in reducing both facultative anaerobes such as *Staphylococcus* spp., *Serratia* spp., *Pseudomonas* spp., and obligate anaerobes including *Clostridiales family XI.* However, in 6/17 samples, there was an increase in bacterial load.

Both facultative and obligate anaerobes were detected together in chronic non-healing DFU samples positive for biofilm presence. Microelectrode and transcriptomic analysis of *in vitro* biofilms have identified the interior of biofilms house niche areas of altered pH levels, oxygen and nutrient depletion.[129,172] Oxygen is noted for its depletion at the substratum layers of biofilms and in the centre of micro-colonies, explaining why wounds complicated by biofilm can harbour such diverse microorganisms. This may indirectly support the action of Cadexomer Iodine against biofilms, as it suggests penetration to deeper areas that house obligate anaerobes are possible, as identified by 16S rDNA of pre-and post- reductions in obligate anaerobes.

Using a combined molecular and microscopy approach can provide an extended understanding of the effects of antimicrobials on wounds. This approach for example provided insights into how Cadexomer Iodine affected microbial populations. Using sample level data, *Serratia* spp. was identified as contributing to 75% of abundance pre-treatment and 0% post-treatment in sample 42 (Figure 5.6). This also correlated with a greater than 1-Log10 reduction in microbial load as determined by real-time qPCR. Similarly, for sample 44, *P. aeruginosa* contributed to 88% of abundance pre-treatment and 0.3% post-treatment, with a 0.76-Log10 reduction in microbial load (Figure 5.7). This suggests that Cadexomer Iodine was effective in reducing the abundant microorganisms in these cases.

Figure 5.6 Pre-and post-community diversity identified through 16S rDNA sequencing. In the left-hand pane sample 42 identifies *Serratia* spp., contributing to 78% abundance pre-treatment and in the right-hand pane this reduces 1% post-treatment. The top of the graph identified the reduction in microbial load by over 1-Log10 reduction determined through qPCR (99% reduction in microorganisms). Therefore, the left-hand pane is a 1% reflection of the remaining microorganisms.

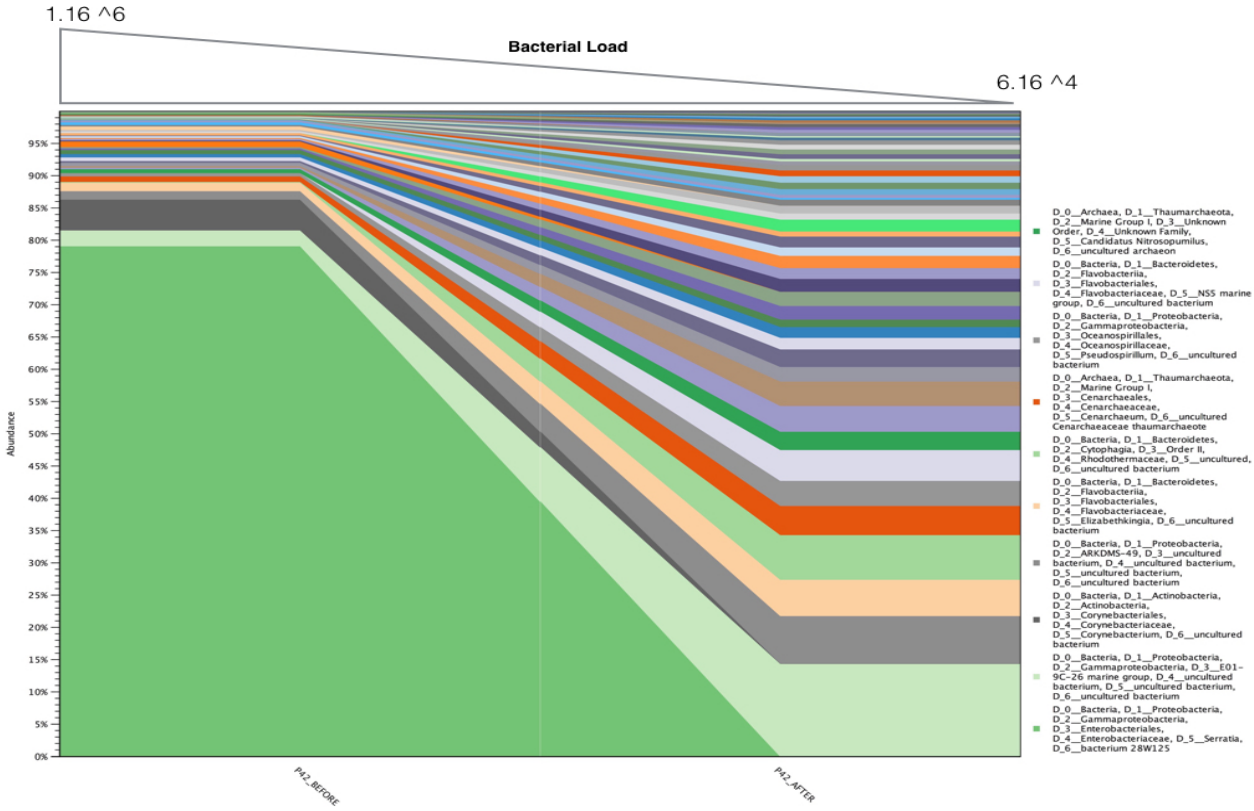
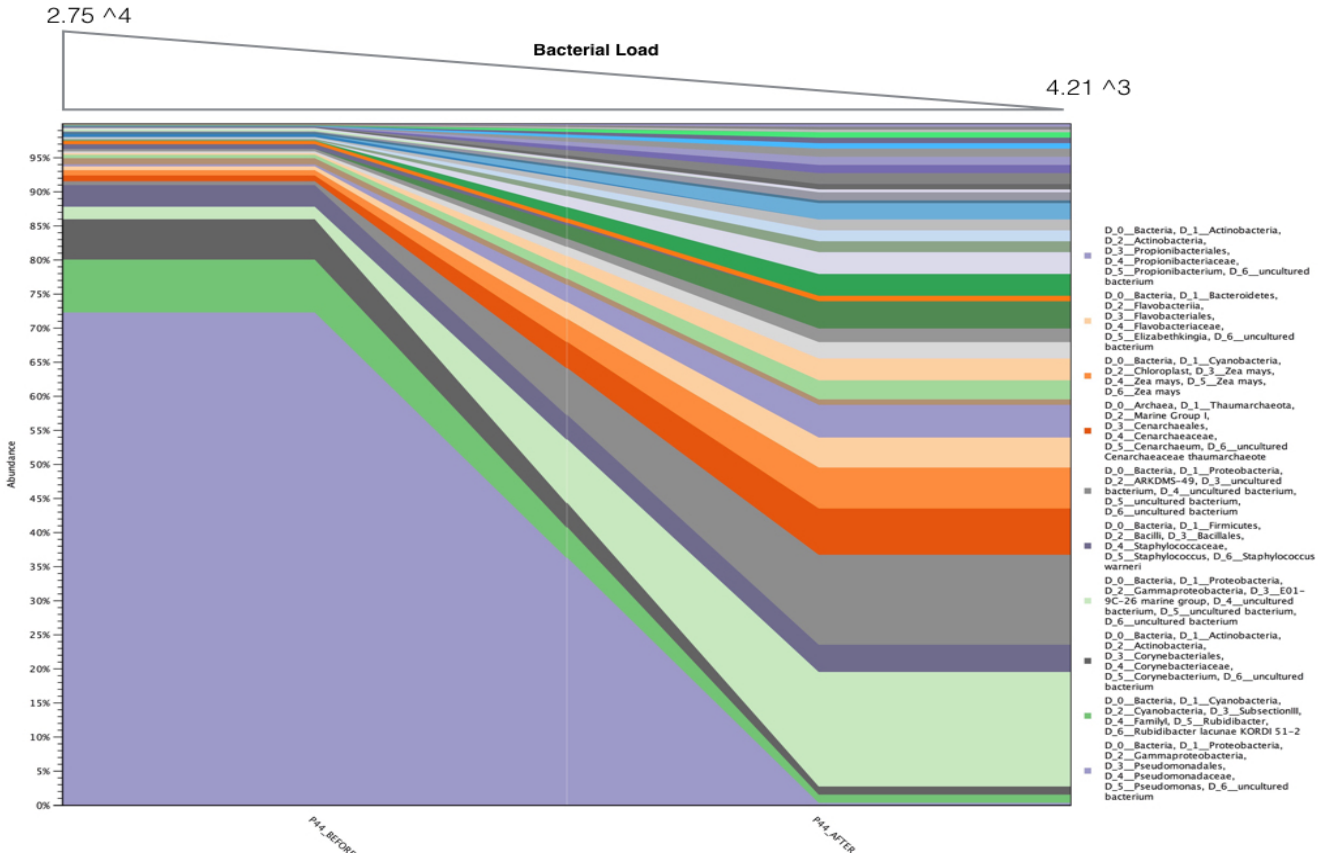


Figure 5.7 Sample 44 identified Pseudomonas spp., Contributing to 78% abundance pre-treatment and 0.3% post-treatment with a 0.76 Log10 reduction.



In contrast, there was no Log10 reduction in microbial load for sample 45 (Pre-treatment = 4.56^5. Post-treatment = 4.73^5). While community profiling revealed a minor reduction in *Staphylococcus* spp., (Figure 5.8) this was countered with a concomitant increase in other microorganisms. In this case, FISH could demonstrate the lack of action against the bulk of microorganisms in sample 45, with pre-and post-images depicting the presence of microbial aggregates as biofilm in chronic non-healing DFU tissue samples (Figure 5.9).

Figure 5.8 Sample 45 identifies a small reduction in *S. aureus*. This is countered by a concomitant increase in other microorganisms allowing an increase in community diversity but not overall microbial load.

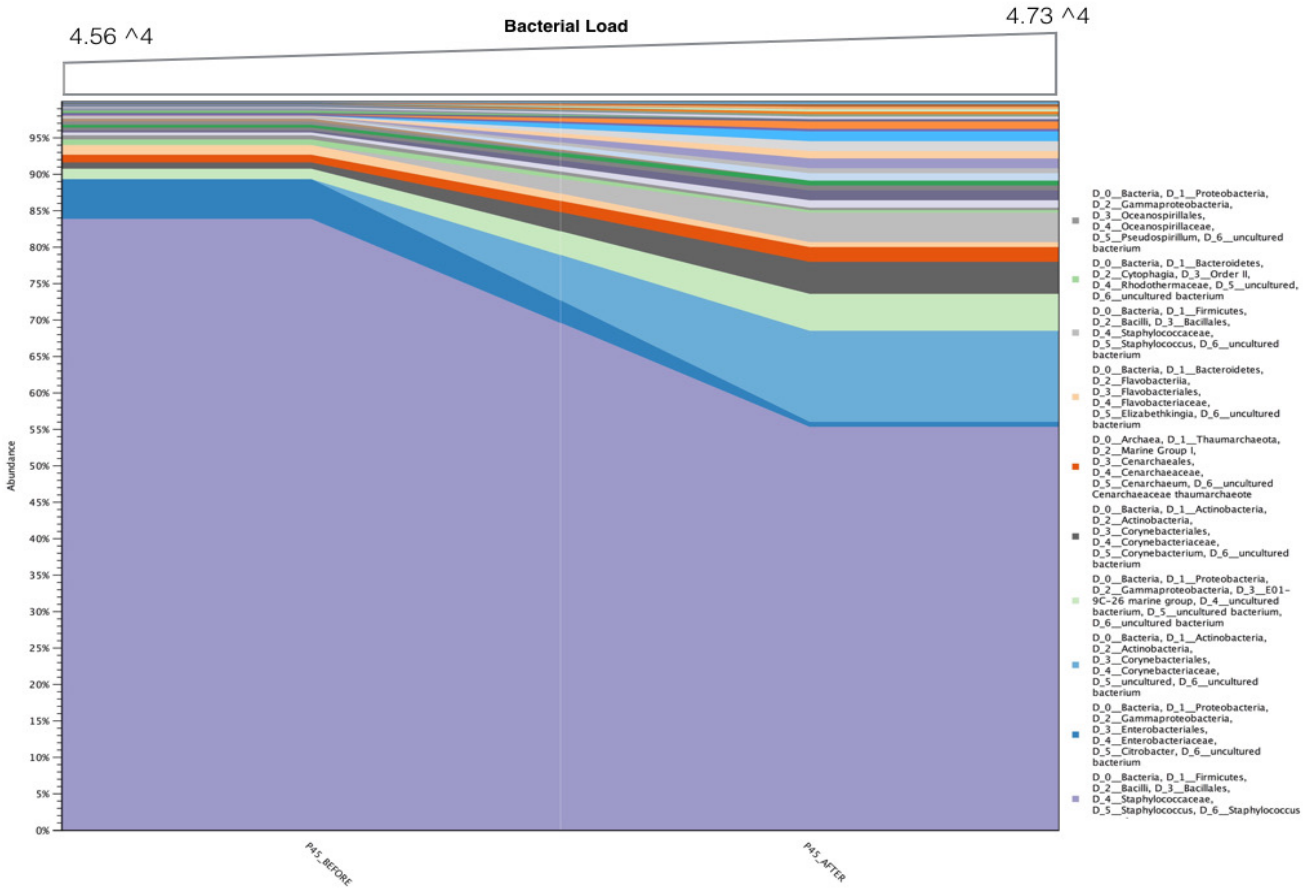
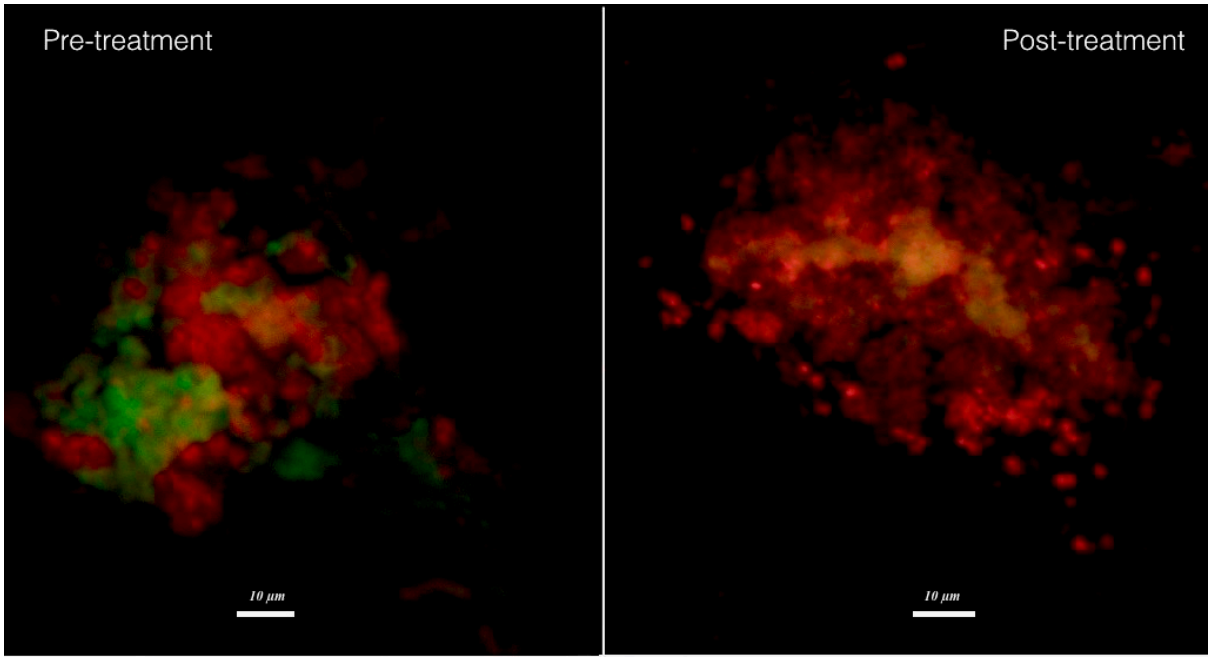


Figure 5.9 FISH image depicting pre-and-post Cadexomer iodine treatment and its effects on microbial aggregates. The red colour represents a universal probe (Cy3), and the green represents a species-specific probe for *S. aureus* (Alexa Fluor 633). Some reduction in *S. aureus* aggregates post-treatment is noted.



In five patients, no Log10 reduction were noted with the use of Cadexomer Iodine. Considering the accepted notion that biofilms are tolerant to antimicrobials and combining the potential attributes of extremophile (and non-extremophile) microorganisms, this may explain why some wound microorganisms were tolerant to treatment. Post-treatment microbiota analysis identified these microorganisms increased in abundance from previously low numbers when skin flora microorganisms such as *Pseudomonas* spp. decreased in abundance following treatment (indicating Cadexomer iodine were effective). This community disruption may have occurred as nutrient availability increased or where mutual benefit arose [14].

In this study we also identified that the mean total MMP-9 and MMP-2 levels reduced following seven days' application of Cadexomer Iodine (only MMP-9 reaching significance $p = .05$). The reduction in protease levels were further correlated to reductions in the microbial load ($p = .03$). Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinase (TIMPs) are a family of proteolytic enzymes secreted by cells involved in wound healing, and their concentrations vary according to the phase of healing [354]. In previous studies of chronic wounds, delayed healing was characterized by an increase in MMPs, and a decrease in tissue inhibitors of metalloproteinases (TIMPs) [355,356]. MMPs also perform multiple roles in the normal immune response to infection and or the presence [357].

MMPs destroy components of the extracellular matrix and damage growth factors and their receptors that are essential for healing [358]. Excessive protease levels which may exist in chronic wounds (either independent or dependent on microbial load) could therefore impede healing [356]. Reducing the microbial load in chronic wounds may have downstream effects on protease levels and this could further contribute to improvements in wound healing, secondary to controlling (reduce, kill or eradicate) biofilm.

Study limitations

The primary aim of this study was to ascertain the effects of Cadexomer iodine on microbial populations associated with the presence of biofilm. Without the controlled conditions afforded by *in vitro* biofilm models, we are not able to fully rule out that planktonic microorganisms contaminated samples. Our rationale to provide a strong argument that planktonic microorganisms were of a negligible proportion of microbial cells, with the bulk of cells being biofilm, is supported by our visualisation techniques using SEM and FISH. These methods identified significant aggregates of microbial cells with EPS. Furthermore, we vigorously

rinsed all tissue samples during preparation as a method to reduce planktonic microorganisms not adhered to tissue. Alternate methods to reduce planktonic microorganisms from contaminating biofilm models have only been eluded to from *ex vivo* animal explants [297], with no reports of this approach on human tissue.

To measure total microbial load, qPCR was utilized as the technique of choice, with previous wound related PCR studies adopting this methodology [67,359]. We acknowledge with this approach the inability of qPCR (based on 16s rDNA) to differentiate live or dead bacteria. Other commonly employed techniques such as molecular viability testing (the pre-RNA analysis) and RT-PCR (viability qPCR) can detect LIVE/DEAD microorganisms. The limitations of this technique for our diabetic ulcer samples are the predominantly biofilm phenotype cells present in the tissue. Pre-RNA rapidly responds to nutrition stimulation and target metabolism, not actual microbial load. Given biofilm cells have low metabolism in comparison to planktonic cells, this may lead to variations in the 16S pre-RNA level between biofilm and planktonic cells and different growth conditions [360]. The log reductions noted in this study therefore represents the minimal response and we acknowledge that some of the bacteria detected by qPCR could be dead, resulting in a lower calculable efficacy for Cadexomer iodine.

Lastly, only eight samples with adequate protein concentration were available for analysis. In the majority obtaining enough wound fluid post treatment was difficult, and this may be associated with reduced wound inflammation with reductions in microbial loads. Alternatively, the time of dipstick applications was undertaken during 30-minute appointments and this may not have been long enough to adequately allow wounds to leak onto the three dipsticks required.

CHAPTER 6

6.1 EVALUATION OF SHORT EXPOSURE TIMES OF ANTIMICROBIAL WOUND SOLUTIONS AGAINST MICROBIAL BIOFILMS: FROM *IN VITRO* TO *IN VIVO*

(APPENDIX 10) JOHANI, K., **MALONE, M.,** JENSEN, S. O., GOSBELL, I. B., DICKSON, H. G., HU, H., VICKERY K. EVALUATION OF SHORT EXPOSURE TIMES OF ANTIMICROBIAL WOUND SOLUTIONS AGAINST MICROBIAL BIOFILMS: FROM *IN VITRO* TO *IN VIVO*. *J ANTIMICROB CHEMOTHER* 2017

Candidate contribution:

- ✓ Worked as senior/corresponding author for publication.
- ✓ Created the study concept and initiated collaboration with University of Florida
- ✓ Contributed to the design of *in vitro* experiment.
- ✓ Designed the study protocol and methodology for *in vivo* study, and performed all aspects of the clinical study, including screening, recruitment, data collection, tissue biopsy sampling and storage.
- ✓ Used genomic software to quality control and analyse DNA sequences from raw files.
- ✓ Used statistical software and knowledge of statistical approaches to analyse / correlate clinical data.
- ✓ Reviewed all SEM and FISH pictures to confirm +ve presence.
- ✓ Wrote the manuscript in full.
- ✓ Submitted the manuscript as senior/corresponding author.

Abstract

Objectives: Test the performance of topical antimicrobial wound solutions against microbial biofilms using *in vitro*, *ex vivo* and *in vivo* model systems at clinically relevant exposure times.

Method: Topical antimicrobial wound solutions were tested under three different conditions; (*in vitro*) 4 % w/v melaleuca oil, polyhexamethylene biguanide, chlorhexidine, povidone iodine and hypochlorous acid were tested at short duration exposure times for 15-minutes against 3-day mature biofilms of *S. aureus* and *P. aeruginosa*. (*ex vivo*) Hypochlorous acid was tested in a porcine skin explant model with twelve cycles of 10-minute exposure, over 24 hours, against 3-day mature *P. aeruginosa* biofilms. (*in vivo*) 4 % w/v Melaleuca Oil was applied for 15-minutes exposure, daily, for seven days, in 10 patients with chronic non-healing Diabetic Foot Ulcers (DFUs) complicated by biofilm.

Results: *In vitro* assessment demonstrated variable efficacy in reducing biofilms ranging between 0.5 log10 reductions to full eradication. Repeated instillation of hypochlorous acid in a porcine model achieved < one log10 reduction (0.77 log10, $p = .1$). Application of 4 % w/v melaleuca oil *in vivo*, resulted in no change to the total microbial load of DFUs complicated by biofilm (median $\log_{10}$ microbial load pre-treatment = 4.9 log10 versus 4.8 log10 ($p = .43$).

Conclusion: Short durations of exposure to topical antimicrobial wound solutions commonly utilised by clinicians are ineffective against microbial biofilms, particularly when used *in vivo*. Wound solutions should not be used as a sole therapy and clinicians should consider multi-faceted strategies that include sharp debridement as gold standard.

6.1.1. INTRODUCTION

Chronic wounds are a serious cause of morbidity and mortality and are associated with reduced patient health-related quality of life. The impacts to health care providers are reflected in the staggering cost of managing these wounds and associated comorbidities, with £5.3 billion attributed to National Health Service expenditure [361]. Increasing evidence about the microorganisms involved in chronic wounds have identified that planktonic cells may not necessarily represent the phenotypic behaviour of microorganisms involved in chronic non-healing wounds. The focus has shifted towards the concept of microbial aggregates (biofilms) that differ markedly in both their phenotypic behaviour, which may contribute to the delayed healing of wounds [141].

In addition, the ecology of chronic wounds explored through molecular DNA based technologies (and not cultivation based methods), have identified these wounds to be complicated by complex polymicrobial communities [90].

Once established, complex biofilm communities often become highly tolerant to standard treatment and removal/eradication paradigms, yielding several hallmark features that distinguish biofilm phenotypes from those of planktonic counterparts. The most notable of these are a remarkable tolerance to antimicrobial agents [145,362] and host immune defenses [146]. The increasing awareness and promotion of biofilm within the wound care arena has led to a dramatic rise in the use of topical antimicrobial solutions as part of wound-care therapeutics [363].

Unfortunately, the evidence for use of particular topical antimicrobials in the treatment of biofilm-associated wounds is based on *in vitro* methodologies that lack standardization and clinical relevance to their intended applications [364]. For example, the anti-biofilm effects of wound solutions/irrigates, where outcomes are based on reductions in biofilm markers (*i.e.*, biomass, cfu/mL, LIVE/DEAD stain viability), have been reported at exposure times far greater than their intended use. Many wound care/device companies promote a 15-minute exposure time of their respective antimicrobial solutions (seconds for irrigates), yet the bulk of data for effectiveness of these products *in vitro* have only reported outcomes at 24-hour exposure times [288,301,365].

This has important consequences at a treatment level where clinicians often seek guidance from laboratory-based studies (due to a lack of available *in vivo* data) in choosing the most relevant and effective agent to reduce microbial biofilms. Therefore, *in vitro* data based on greater exposure times may not reflect the most clinically appropriate outcomes for clinicians using these products at shorter exposure times. This is highlighted succinctly by Castaneda *et al* (2016) [303], who showed that in an *in vitro* biofilm model, antimicrobial susceptibility increased with antimicrobial exposure time.

The present study was designed to explore if shorter durations of exposure of antimicrobial wound solutions were effective against microbial biofilms (i) *in vitro* against mature biofilms of *S. aureus* and *P. aeruginosa* (ii) in an *ex vivo* porcine skin explant model against mature *P. aeruginosa* biofilms and (iii) *in vivo* in 10 patients with chronic non-healing Diabetic Foot Ulcer (DFUs).

6.1.2. MATERIALS AND METHODS

Bacteria

Biofilm forming reference strains utilized *in vitro* were *S. aureus* (ATCC® 25923™, (methicillin-sensitive *S. aureus*—MSSA) and *P. aeruginosa* (ATCC® 25619™), and *P. aeruginosa* PA01 (ATCC® BAA-47™) was used in the *ex vivo* porcine skin explant model.

Antimicrobial wound solutions

The solutions/irrigates examined, any incorporated antimicrobials/antiseptics and their respective manufacturers were as follows: (SBMO) Surfactant based antiseptic solution with 4 % w/v Melaleuca Oil (Woundaid® Woundwash, Mundipharma, Singapore), (SBPHMB) Surfactant based antimicrobial solution with polyhexamethylene biguanide (Prontosan®, B Braun Medical, Melsungen, Germany), (SOS) Superoxidized solution contains sodium hypochlorite, hypochlorous acid, sodium chloride, and oxidized water (Microcyn®, Oculus Technologies of Mexico), (CHX) Chlorhexidine 4.5mg/30mL (0.015% w/v) and cetrimide 45mg/30mL (0.15% w/v) irrigation solution, (Pfizer, New York, United States), (PVP-I) Povidone iodine antiseptic solution 10%w/v equivalent to 1% w/v available iodine (BETADINE®, Mundipharma, Singapore), NaCl 0.9% (Baxter, Illinois, United States).

The decision to use SOS for the *ex vivo* porcine explant model and SBMO for human i*n vivo* study was based on clinical relevance. Both the use and promotion of these "newer generation" solutions with antimicrobial properties (as opposed to traditional antimicrobials of CHX and PVP-I) by clinicians and industry for action against wound biofilm has increased significantly over the last decade. They now represent the predominant products used for wound cleansing and debridement.

Experimental models

In vitro model

Biofilm, containing 10^7 – 10^8 cells/coupon of *P. aeruginosa* (ATCC 25619) and 10^6 cells/coupon of *S. aureus* ATCC 25923 were grown under shear (130 r.p.m.) on polycarbonate coupons in a CDC biofilm reactor (BioSurface Technologies Corp., Bozeman, MT) as previously described by our group [306], in 400 mL of 15 g/L (50%) Tryptone Soy Broth (TSB) (Sigma Aldrich, St. Louis, MO, United states) at 35 °C in batch phase for 48 hours, followed by fresh media (20% TSB, 6 g/L) for a further 24 hours. Coupons were washed in 10 mL phosphate buffered saline (PBS) to remove loosely attached planktonic bacteria. Each coupon had 10^7 – 10^8 *P. aeruginosa* or 10^6 *S. aureus.*

Five antiseptic treatments were tested (SBMO, SBPHMB, SOS, CHX, PVP-I); four coupons were exposed to each treatment condition for 15 minutes, while an additional four coupons were used as controls. The numbers of bacterial colony forming units (cfu) per coupon were tested in triplicate by sonication in an ultrasonic bath (Soniclean, Thebarton, Australia) for 10 minutes with a sweeping frequency of 42-47 kH at 20oC. The coupon was then vortexed for 2 minutes in 2 mL of PBS followed by sequential 10-fold dilution and plate count. Pre-and post-exposure average cfu/ coupon was expressed as log10.

Bacterial cell viability pre-and post-exposure was also assessed using *Bac*Light™ (Live/Dead Bacterial Viability Kit, 7012, Molecular Probes, Invitrogen, Carlsbad, CA, United States) in conjunction with confocal laser scanning microscopy (CLSM) and expressed as percentage viability as determined by Imaris (v 8.4, ImarisXT, Bitplane). For confocal, we used inverted laser scanning confocal microscope LSM 880 (ZEISS LSM 880, Carl Zeiss Ltd., Herefordshire, UK) for all the samples, with oil-immersion lenses (63x and

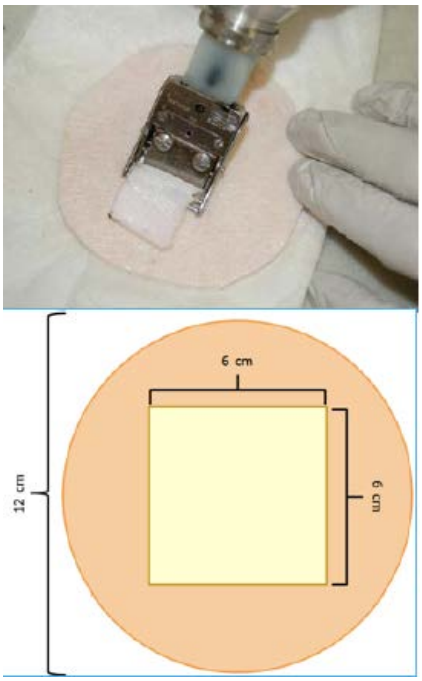
100x) and acquisition parameters of frame Size: 1024 x 1024, Speed:6, Averaging: 2, Bit Depth: 12.

Ex vivo porcine skin explant model

The *ex vivo* porcine skin explant biofilm model used in this study is previously described [366]. Large sheets of fresh pig skin (approximately 30 cm x 30 cm) were obtained from a USDA approved commercial meat processing lab. The skin was thoroughly cleaned, and the hair was closely trimmed using an electric clipper and safety razor. The subcutaneous fat layer was trimmed away so that only approximately 1-2 mm thickness remained. The pig skin was then processed based on previous results of experiments assessing KCI VAC-Ulta® therapy (Acelity Inc., San Antonio, TX, United States) on porcine skin biofilm explants.

Briefly, a circle shape approximately 12 cm in diameter was traced on the dorsal surface sheet of the large, cleaned porcine skin using a marking pen. A partial thickness excision wound measuring 6 cm x 6 cm (36 cm^2) and 0.8 mm deep was made in the center of each circular explant using an electric Paget dermatome. The circular explants were then cut from the large sheet of pig skin using heavy scissors (Figure 6.1).

Figure 6.1 Method for creating partial thickness injuries to porcine skin.



Explants were sterilized by first submerging the explants in PBS containing 0.6% hypochlorous acid and 0.5% Tween 80 for 5 minutes then transferred to a chlorine gas chamber for 45 minutes, followed by submerging the explants again in PBS containing 0.6% hypochlorous acid and 0.5% Tween 80 for 5 minutes. The sterile explants were rinsed twice in sterile PBS then transferred into 150 mm diameter x 25 mm deep culture plates (176 cm^2 surface area) (Corning 430599) containing 0.5% tryptic soy soft agar containing antibiotic (gentamicin at 50 mg/mL) to limit planktonic growth and promote biofilm growth on the explants. Partial thickness injuries measuring approximately 6 cm x 6 cm x 0.8 mm deep were created using an electric Paget dermatome to centre of a circular pig skin explant approximately 12 cm in diameter. After sterilization with hypochlorous acid and chlorine gas, explants were placed into 150 mm diameter sterile culture plate containing 0.5% soft tryptic soy agar with antibiotic then inoculated with P. aeruginosa and grown for 3 days at 37ºC.

Growth of mature P. aeruginosa biofilm on sterile pig skin explants

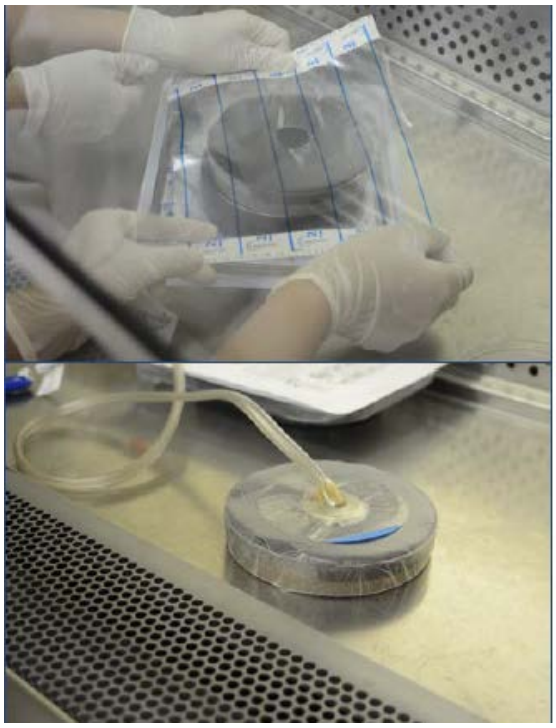
The wound area on each sterile pig skin explant was inoculated with 100 µl of planktonic culture containing approximately $10^7 - 10^8$ colony forming units per milliliter (cfu/mL) of *P. aeruginosa* bacteria. The suspension culture of *P. aeruginosa* planktonic bacteria was in early log phase growth (0.2 -0.6 OD_{640nm}) and was serially diluted in phosphate buffered saline (PBS) (4.5 mL) and plated in triplicate (0.1 mL) onto tryptic soy agar (TSA) to determine starting cfu/mL of planktonic culture.

The *P. aeruginosa* inoculated explants were incubated for 3 days at 37°C in an atmosphere of 5 % CO_2 in air saturated with water vapor. The explants were transferred daily to fresh sterile 0.5% soft TSA supplemented with antibiotic. After the three days of growth on TSA soft agar, the explants with the mature biofilm were rinsed with sterile PBS then transferred into fresh 150 mm diameter x 25 mm deep culture plates (176 cm^2 surface area) (Corning 430599) with a base of TSA supplemented with antibiotic. Any excess PBS was removed from the surface of the explants by gentle aspiration.

Treatment of biofilms on pig skin explants with V.A.C. Ulta™ instillation system

Application of the V.A.C. Ulta™ therapy system (Acelity Inc., San Antonio, TX, United States) to pig skin biofilm explants required two people. The appropriate sized V.A.C. VeraFlow™ Dressing (Acelity Inc., San Antonio, TX, United States) was applied to the surface of the porcine explants to cover the explant and 'wound' area and a V.A.C. VeraT.R.A.C™ Duo Tubing Set (Acelity Inc., San Antonio, TX, United States) was applied over the VeraFlow™ dressing (Figure 6.2).

Figure 6.2 Application of the V.A.C. VeraT.R.A.C™ Duo Tubing Set to the Pig Skin Explant.The appropriate sized V.A.C. VeraFlo™ dressing is applied to the surface of the porcine explants to frame the 'wound' area. The drape is pulled and pressed firmly over the edge of the culture dish to ensure an air-tight seal between the dish and the V.A.C. Ulta™ pad during treatment.



The drape was pulled and pressed firmly to the surface of the framed pig skin and the edge of the culture dish to ensure an air tight seal to the dish. The V.A.C. VeraT.R.A.C™ Duo Tubing Set was connected to the canister and irrigation tubing respectively and negative pressure (125 mm Hg) with instillation of appropriate solutions was applied using the V.A.C.Ulta™ according to the conditions described below in the four test groups. The V.A.C.Ulta™ therapy for this experiment consisted of twelve (12) cycles of two (2) hours duration for each cycle for a total of 24 hours of the experiment. Each cycle consists of instillation of 75 mL of fluid per cycle, 10 minutes of soak/dwell time followed by 110 minutes of continuous negative pressure.

Test conditions for Experiment 1:

1. Control negative pressure only - 12 cycles of intermittent negative pressure, no instillation
2. Microcyn -- 12 cycles of V.A.C. Ulta^{TM} with instillation of SOS at 10-minute dwell
3. 0.125% bleach -- 12 cycles of V.A.C. Ulta^{TM} with instillation of bleach solution at 10-minute dwell
4. Saline -- 12 cycles of V.A.C. Ulta^{TM} with instillation of saline solution at 10-minute dwell.

Quantitative assessment of biofilm bacteria surviving on pig skin explants

After 24 hours of treatment (12 treatment cycles), the drape and dressing were removed, and six biopsies of 8 mm diameter weretaken aseptically from the wound area of the explant at the six points of a hexagon pattern (60 degrees of angle separation) covering the wound bed under the V.A.C.Ulta^{TM} pad (Figure 6.3). The six biopsies were transferred to separate tubes containing 5 mL of sterile PBS then sonicated in a water bath for 5 cycles of 1.5 minutes of sonication with a 1-minute pause between sonication cycles. The bacterial suspension was serially diluted and plated in triplicate onto TSA plates to measure the number of colony forming units of biofilm bacteria after 24 hours of culture at 37°C. Two additional biopsies were taken from the center of the wound bed under the V.A.C. Ulta^{TM} pad and one was processed for scanning electron microscopy (SEM) using standard glutaraldehyde fixation and sputter coating and one biopsy was processed for light microscopy by formaldehyde processing with paraffin embedding H&E staining.

Figure 6.3 Biopsies obtained from the centre of the explant - one for SEM and one for light microscopy.

In vivo clinical study

We used a combined molecular and microscopy approach described previously[334] to better understand the effects of a topical antimicrobial solution against the microbial load and diversity of chronic non-healing DFUs complicated by biofilm Ten patients with chronic non-healing DFU (and not on current antimicrobial therapy) were enrolled over a six-month period from a tertiary referral hospital (Liverpool Hospital High Risk Foot Service, Liverpool, Sydney). Ethics approval for this study was granted by the South West Sydney Local Health District Research and Ethics Committee (HREC/14/LPOOL/487, SSA/14/LPOOL/489). SBMO was soaked in sterile gauze and applied to the wound for 15 minutes, every day for seven days.

Sharp debridement of tissue was withheld over the seven-day treatment period, as this would have likely affected the primary outcome measure [309]. Tissue punch biopsies were obtained from the wound edge for each participant after cleansing the wound with NaCl 0.9% pre-and post-treatment. These were subjected to qPCR to determine the total microbial load, 16S rDNA next generation sequencing to explore the microbiota of chronic DFUs and the effect of topical antimicrobial therapy on microbial communities, scanning electron microscopy (SEM) to visualize biofilm structures and fluorescent in situ hybridisation (FISH) in conjunction with CLSM to examine spatial organisation of microbial aggregates.

Specimen collection and storage for in vivo analysis

Tissue biopsies were obtained from the wound edge for each participant after cleansing the wound with NaCl 0.9%. Tissue samples for DNA analysis were placed immediately into RNA*later*® (Ambion, Inc) for 24 hours at 4°C and then frozen at -80°C until DNA extraction. Tissue samples for FISH were immediately fixed in a 4% paraformaldehyde overnight at 4°C, then transferred into PBS and frozen at -80°C. An additional tissue punch biopsy was obtained for SEM (if possible) and immediately fixed in 3% glutaraldehyde overnight at 4°C, then transferred into 0.1% phosphate buffer (PB) and frozen at -80°C.

qPCR to determine microbial load in DFU biofilms

We utilised real-time quantitative PCR (qPCR) using the 16S rDNA eubacterial universal primers 341F 5'-CCTACGGGAGGCAGCAG-3' and 534R 5'-ATTACCGCGGCTGCTGG-3'to determine microbial load in DFU biofilms as previously reported [322,323]. The total number of bacteria was expressed as per mg of tissue normalised to the average number of copies of the 18S rDNA gene in a mg of human tissue.

16S rDNA Next generation DNA sequencing of DFUs

Genomic DNA was extracted from 5-10 mg of DFU biopsy sample using Mo Bio PowerBiofilm DNA isolation kit (Mo Bio Cat# 24000-50) following the manufacturer's instructions. DNA sequencing was carried out by a commercial laboratory (Australian Centre for Ecogenomics, Brisbane, Australia) targeting the V3-V4 region of the 16S rDNA using eubacterial universal primers 515F and 806R [315]. Preparation of the16S rDNA library was performed using the workflow outlined by the manufacturer (Illumina, San Diego, CA, United States).

In the 1st stage, PCR products were amplified according to the specified workflow with an alteration in polymerase used to substitute Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, Massachusetts, United States). Resulting PCR amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, United States). Purified DNA was indexed with unique 8bp barcodes using the Illumina Nextera XT 384 sample Index Kit A-D (Illumina, San Diego, CA, United States) in standard PCR conditions with Q5 Hot Start High-Fidelity 2X Master Mix. Indexed amplicons were pooled in equimolar concentrations and sequenced on the Illumina MiSeq platform using paired end sequencing with V3 300bp chemistry.

Sequence analysis and quality control

Sequence quality control and analyses were performed using QIIME [367]. This is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data. Taxonomy assignment was aligned against the Greengenes reference database (version 2013/05) at 97% similarity. Statistical analyses and data mining were performed using Calypso software 5.8 (http://cgenome.net). Where OTUs of interest (such as those previously cited in

infection or delayed healing) were assigned at only the genera level, these sequences were further referenced for speciation using NCBI Mega BLASTtn [368]. For some OTUs, it was not possible to identify to species level. This is a commonly cited limitation when using hypervariable regions of 16S rDNA sequencing [369].

Fluorescent in situ hybridisation (FISH) with CLSM

Biopsy material was embedded in optimal cutting temperature (OTC) embedding matrix (Fisher Scientific, Waltham Massachusetts, United States), frozen at –80ºC, cryo-sectioned to a thickness of 6 μm and mounted on SuperFrost Plus slides (Menzel-Glaser, Lomb Scientific, Australia). Different types of probes were utilized for in situ hybridization as previously described [335]. PNA-FISH, probes and kits were sourced commercially (AdvanDx, Inc., Woburn, MA, United States) using previously described methods [13].

Briefly, species-specific *Staphylococcus aureus*/coagulase-negative *Staphylococci* (CNS) probes were used. The hybridization solution, was added drop-wise to each tissue section and hybridized at 55°C for 90 min. The slides were washed for 30 min at 55°C in wash solution. Once dry, the coverslip was mounted using a single drop of mounting medium. Slides were examined using CLSM (Zeiss Axio Imager Microscope and/or ZEISS LSM 880, Carl Zeiss Ltd., Herefordshire, UK). Images were processed using ZEISS ZEN Imaging Software (black edition) and Imaris v 8.4, ImarisXT, Bitplane.

Scanning Electron Microscopy (SEM) and image interpretation

DFU biopsy samples were fixed in 3% glutaraldehyde, followed by 3 washes of 0.1M phosphate buffer prior to serial ethanol dehydration and hexamethyldisilazane incubation

(Polysciences, Inc., Warrington, Pa, United States) as described previously [338]. Dried samples were coated with 20-nm gold film in a sputter coater and examined in a scanning electron microscope. Each sample was scored based on the amount of bacteria/biofilm observed using an arbitrary five-point scale where score 0 = no bacteria observed; score 1 = single individual cells; score 2 = small micro-colonies (~ 10 cells); score 3 = large micro-colonies (~100 cells); score 4 = continuous film; score 5 = thick continuous film [339]. Each tissue sample was viewed under SEM averaging two hours per sample. Tissue was screened for microbial aggregates and extracellular polymeric substances (EPS) from the wound surface downwards, working in a zigzag pattern at magnifications ranging from 300X to approximately 5,500X.

Characterization and visualization of DFU biofilm in vivo

The presence or absence of biofilms in DFUs were confirmed through SEM or PNA-FISH. For the purpose of the study, the definition of biofilm was "microbial aggregates surrounded by a self-produced or host derived matrix adhering to natural or artificial surfaces in the host, or aggregates associated with but not directly adherent to the surface" [93].

Statistics

Mann-Whitney U tests were used to assess differences between pre-and post log10 cfu using Statistical Package for Social Sciences Version 23 (SPSS Inc., Chicago, Illinois, USA). CLC genomics workbench version 8.5.1 in combination with the microbial genome-finishing module (CLC bio, Qiagen Aarhus, Denmark) were used to analyse DNA sequence data. QIIME was utilised to visually represent data. Analysis of variance (ANOVA) and permutational analysis of variance (PERMANOVA) were used for statistical analysis of alpha and beta diversity measures. Principal coordinates analysis (PCoA) plots with Bray–Curtis distances were used to assess how dissimilar microbial communities were pre-and-post-treatment.

Community richness of DFUs was presented using richness index reporting the number of unique OTUs in each wound sample. Shannon Weaver Index is an ecological measure of diversity that includes the number of unique microbial taxa and their relative evenness within each sample. For all comparisons and modelling, the level of significance was set at $p < 0.05$. Data are given as mean, median and standard deviation (±) and 95% confidence interval (CI).

6.1.3. RESULTS

Antimicrobial efficacies of topical wound solutions/irrigates against mature biofilms in vitro

The effects of topical antimicrobial solutions on reductions in log10 cfu/coupon following treatments at 15 minutes and 24 hours are reported (Figures 6.4 and 6.5). Live/Dead stain with CLSM and the percentage of red signal (dead/damaged cells) and green signal (viable cells) at 15-minute exposure is noted in Figure 6.6. At 15-minute exposures PVP-I was the only solution to show complete and efficient killing of both *S. aureus* and *P. aeruginosa* biofilms (6 log10 and 7 log10 reduction, $p < .001$). CHX was effective against *S. aureus* biofilms showing complete removal of all bacteria (6 log10 reduction, $p < .001$), and further demonstrated a ≥4 log10 cfu reduction against *P. aeruginosa* biofilm (3.96 log10, $p = .01$).

In contrast, SOS demonstrated complete eradication of *P. aeruginosa* biofilm (7 log10 reduction, $p < .001$), and a ≥4 log10 cfu/mL reduction against *S. aureus* (4.3 log10 reduction $p = .01$). No significant reduction in *S. aureus* counts was observed for treatment with SBPHMB (0.8 log10 reduction), however it was highly effective against *P. aeruginosa* biofilm showing complete eradication (7 log10 reduction, $p = .01$). Treatment with SBMO was ineffective against both *S. aureus* and *P. aeruginosa* biofilm. In contrast, treatment of biofilm with topical

antimicrobials for 24-hours exposure showed complete and efficient killing of biofilm, except for SBMO that failed to eradicate *S. aureus* (but still achieved a ≥2.5 log10 cfu/coupon).

Figure 6.4 Bars represent means of logarithms of colony-forming units of viable biofilm cells after 15-minute exposure.

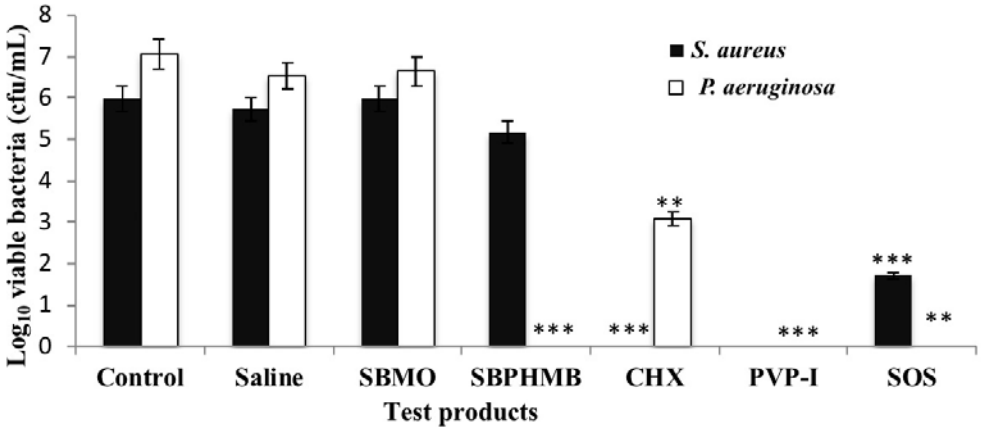


Figure 6.5 After 24-hours exposure. Error bars represent the standard error of the means from three coupons (***p* = .01, *** p < .001 – no viable cells).

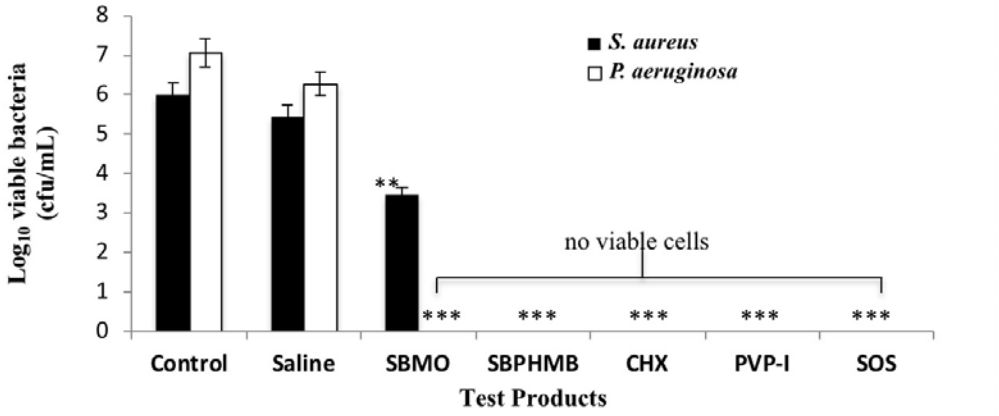
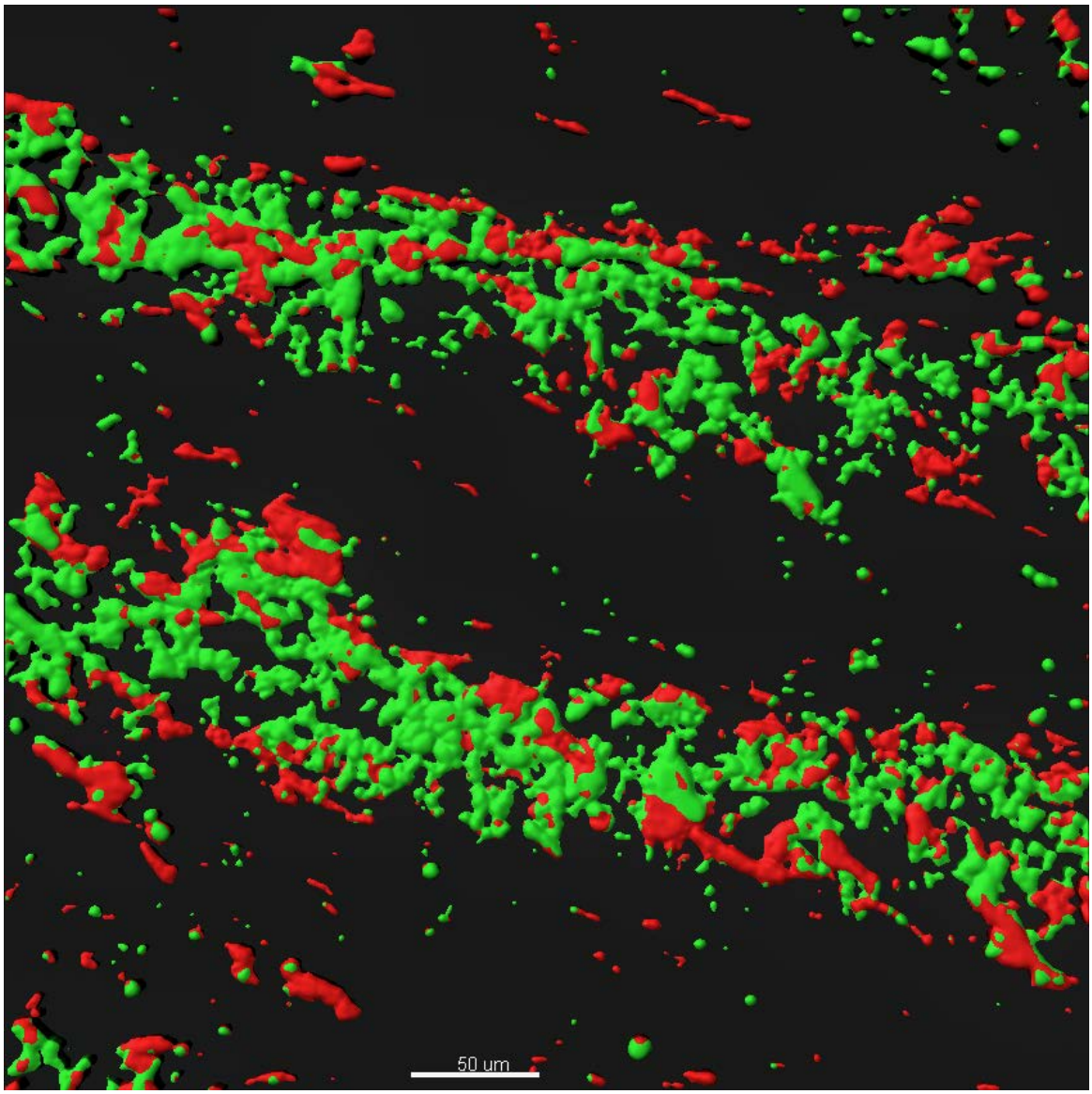
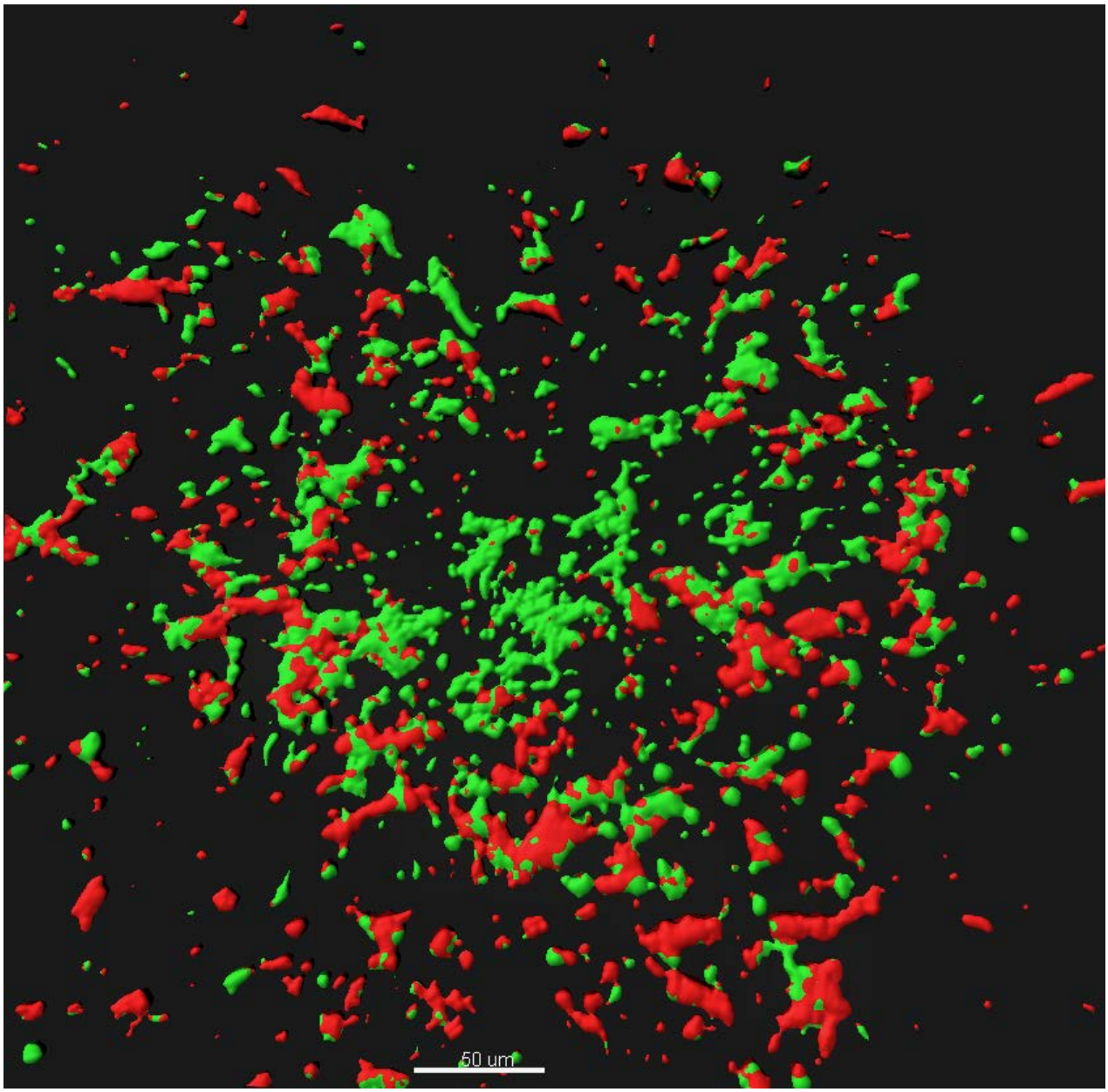


Figure 6.6 Live/Dead stain with CLSM at 15-minute exposure to topical antimicrobial solutions. Percentage of red signal (dead/damaged cells) and green signal (viable cells) determined by the Imaris software (v 8.4, ImarisXT, Bitplane)

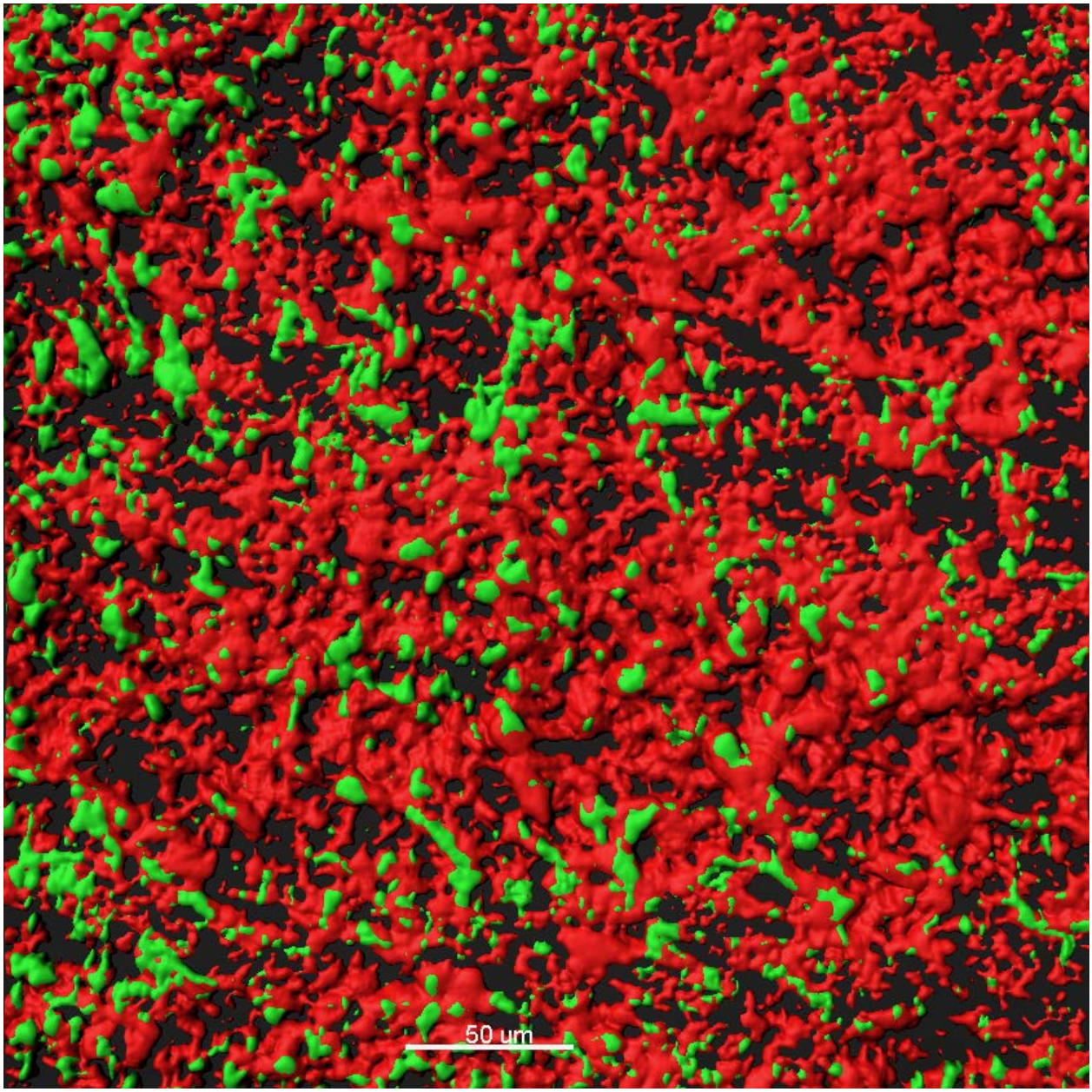
(a) SBMO against *S. aureus* (ATCC® 25923™)



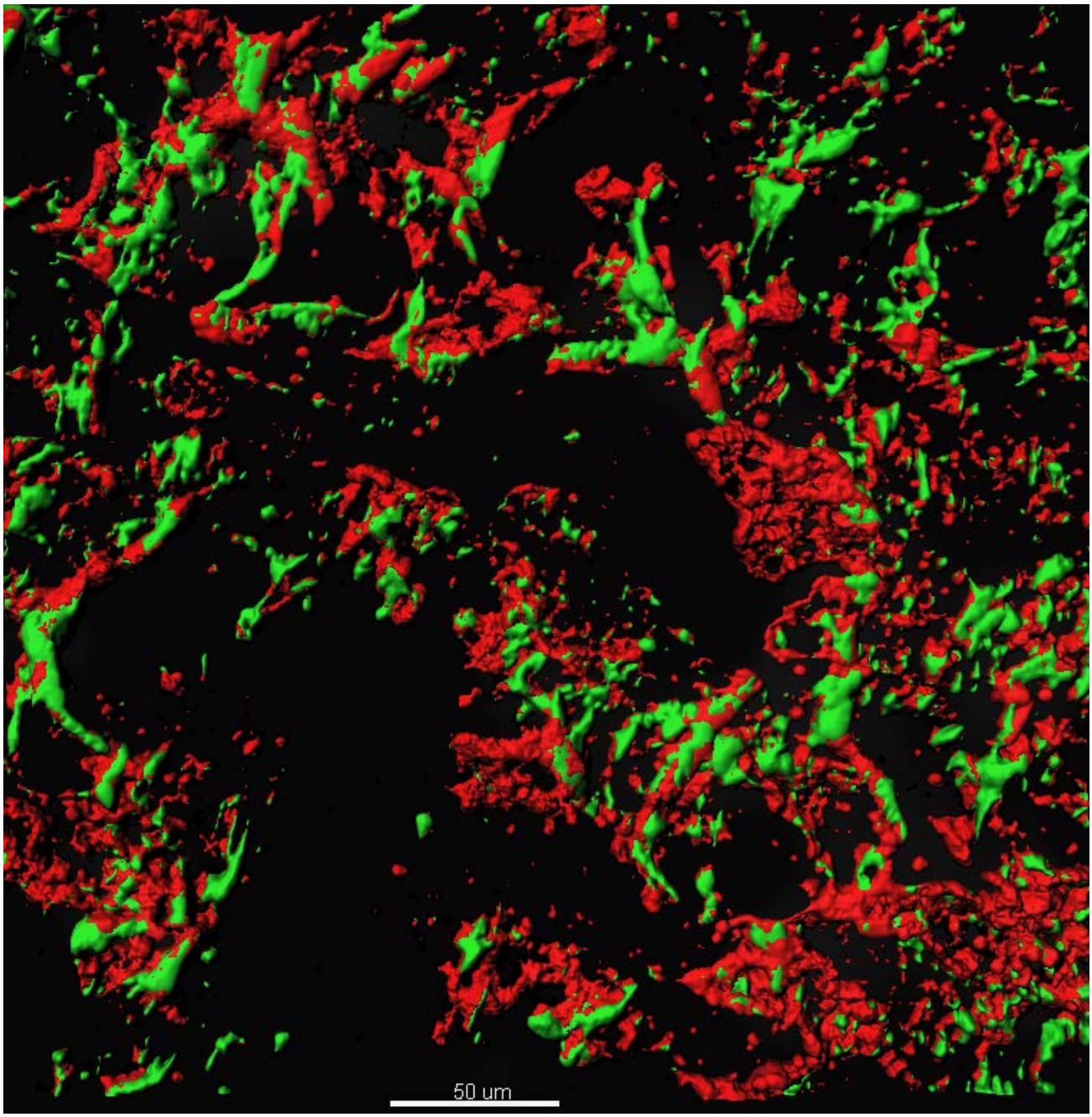
(b) SBMO against *P. aeruginosa* (ATCC® 25619™)



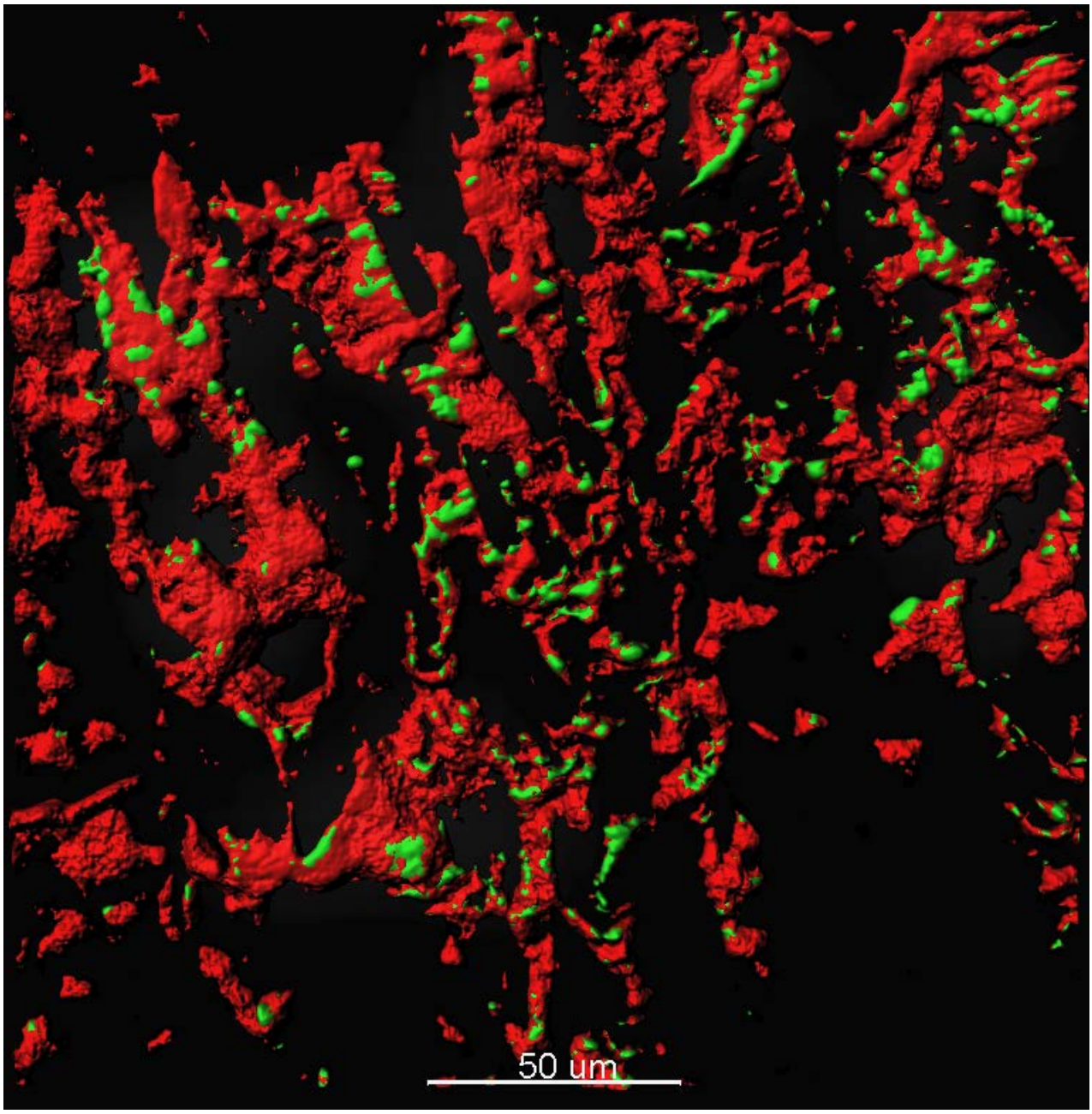
(c) SBPHMB against *S. aureus* (ATCC® 25923™)



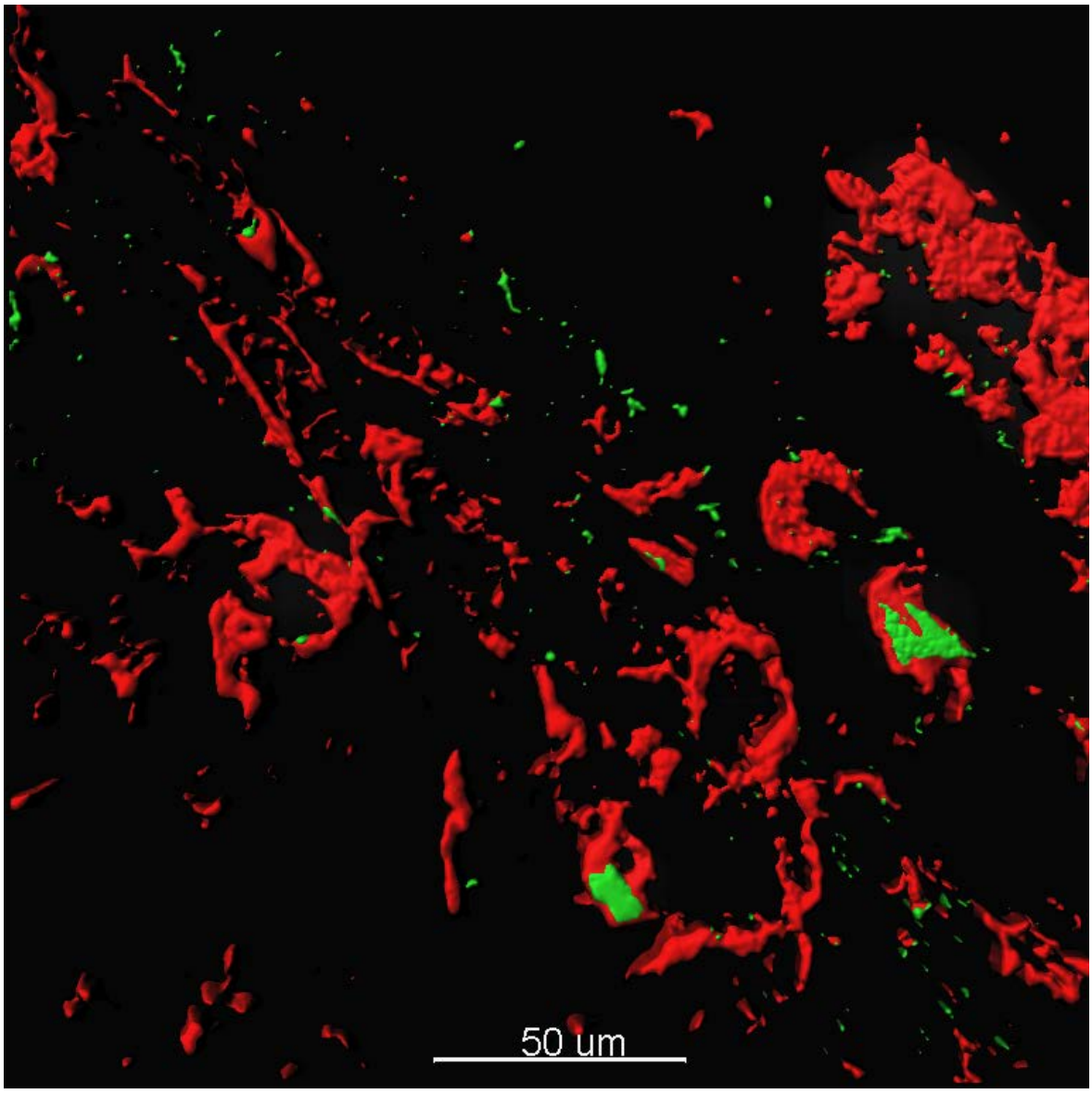
(d) SBPHMB against *P. aeruginosa* (ATCC® 25619™)



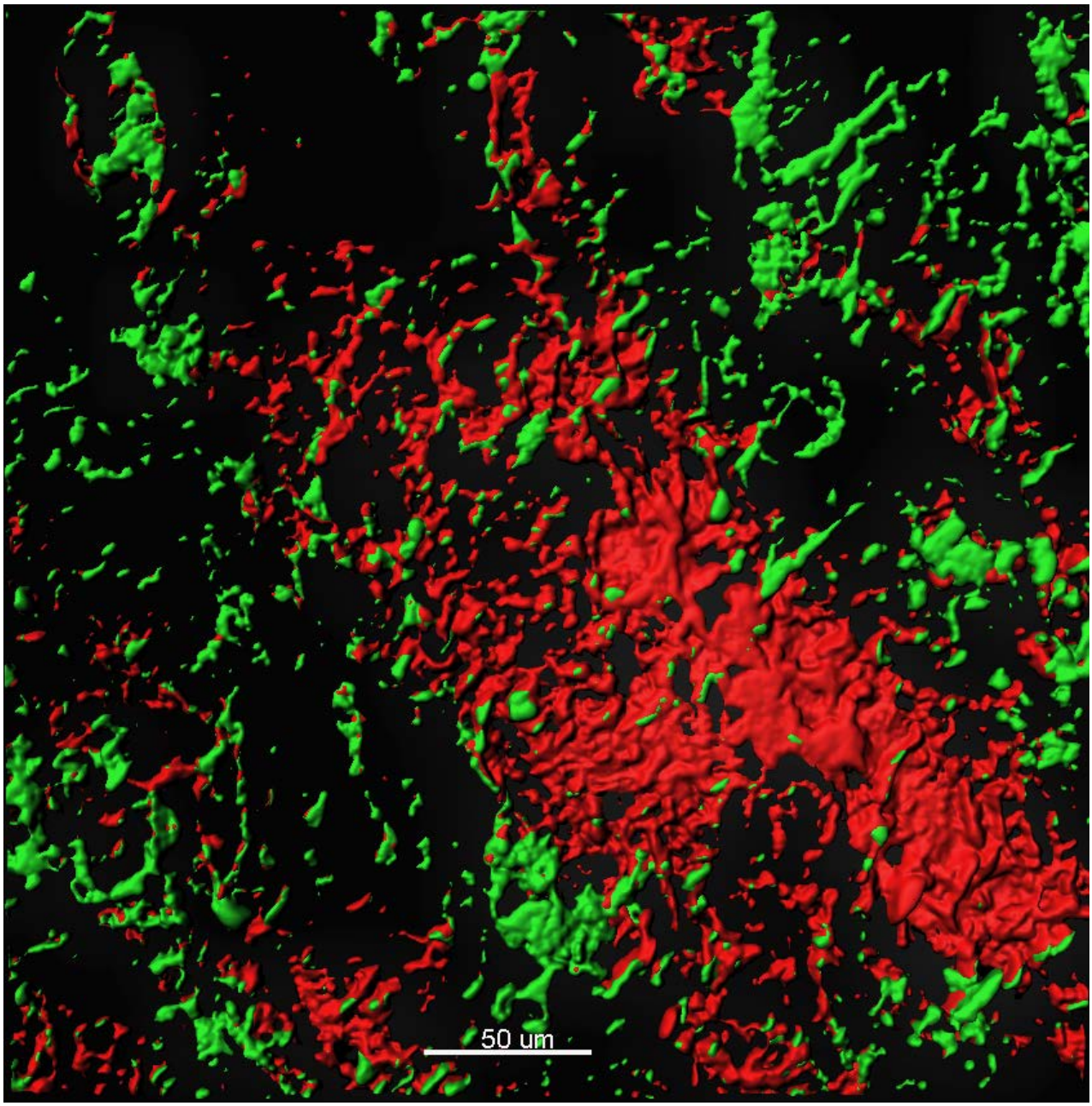
(e) CHX against *S. aureus* (ATCC® 25923™)



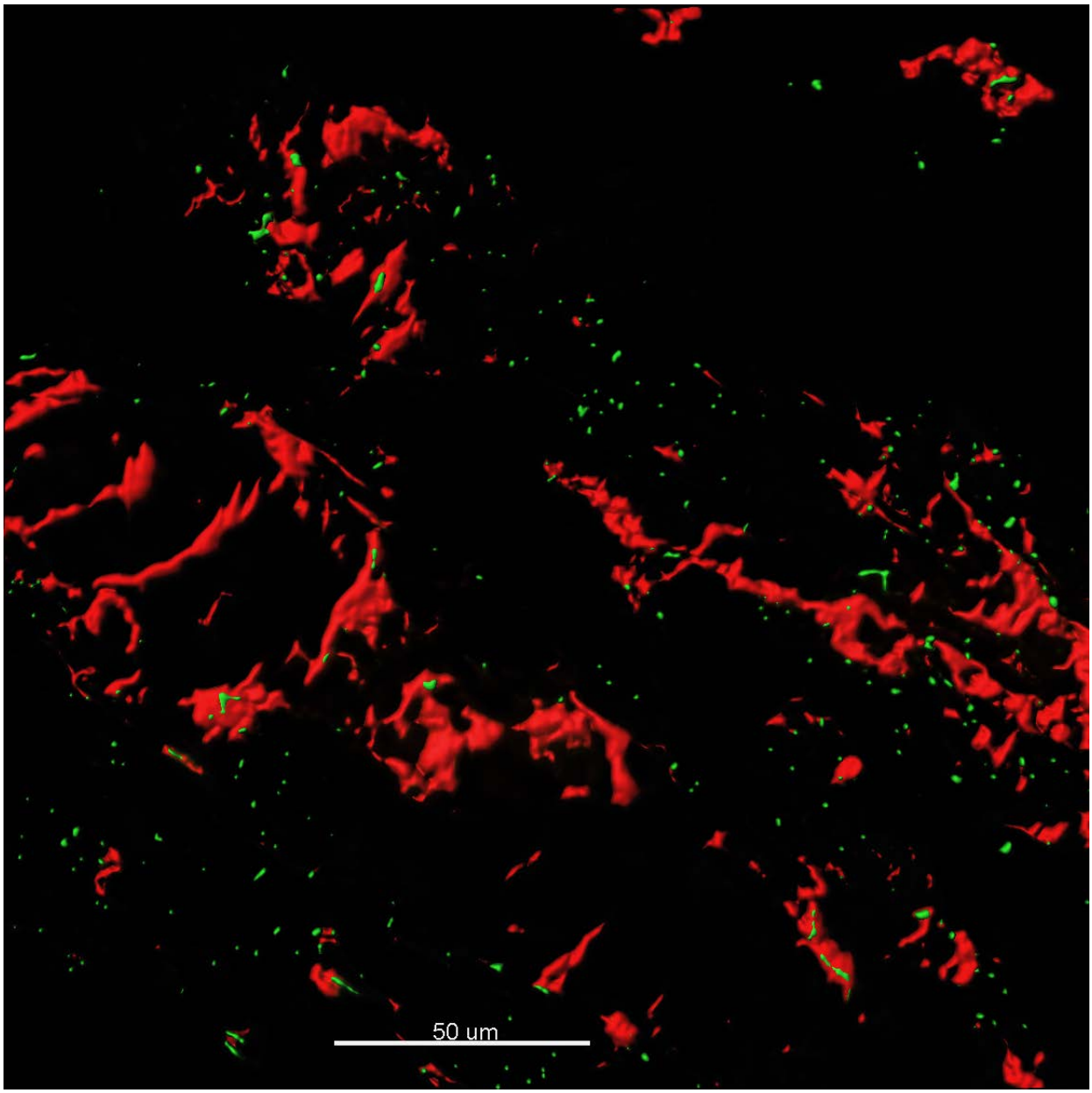
(f) CHX against *P. aeruginosa* (ATCC® 25619™)



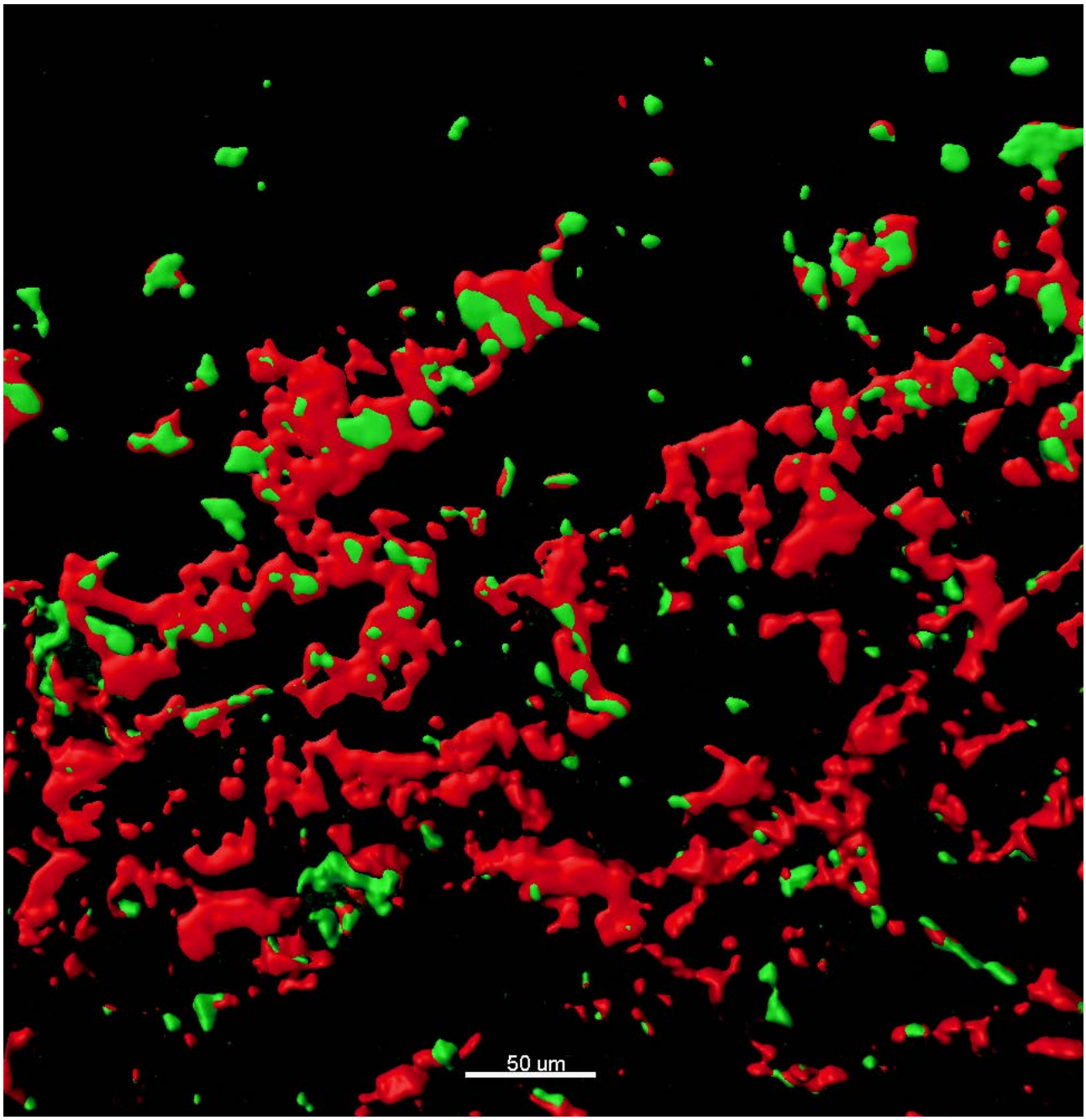
(g) PVP-I against *S. aureus* (ATCC® 25923™)



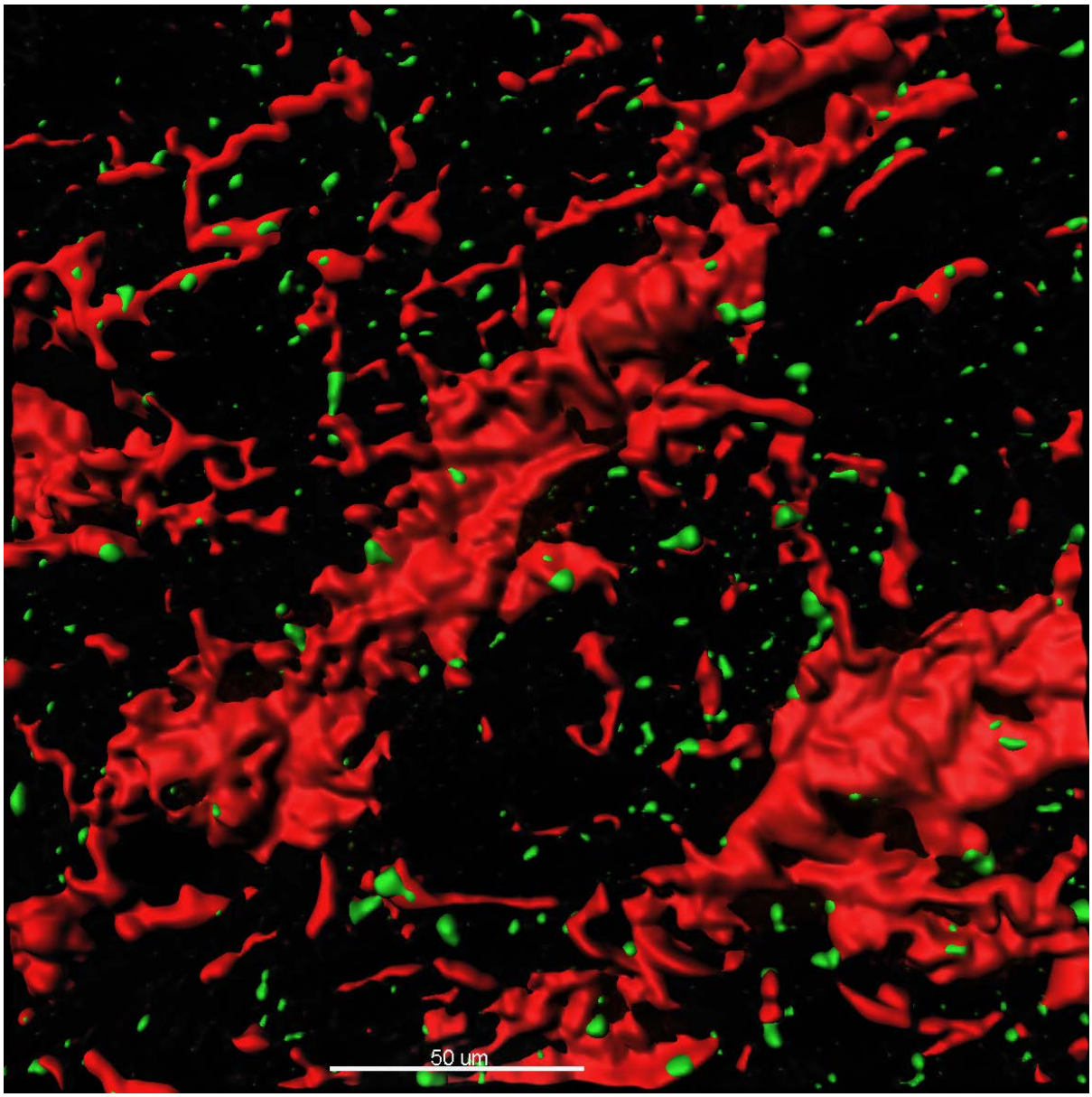
(h) PVP-I against *P. aeruginosa* (ATCC® 25619™)



(i) SOS against *S. aureus* (ATCC® 25923™)



(j) SOS against *P. aeruginosa* (ATCC® 25619™).



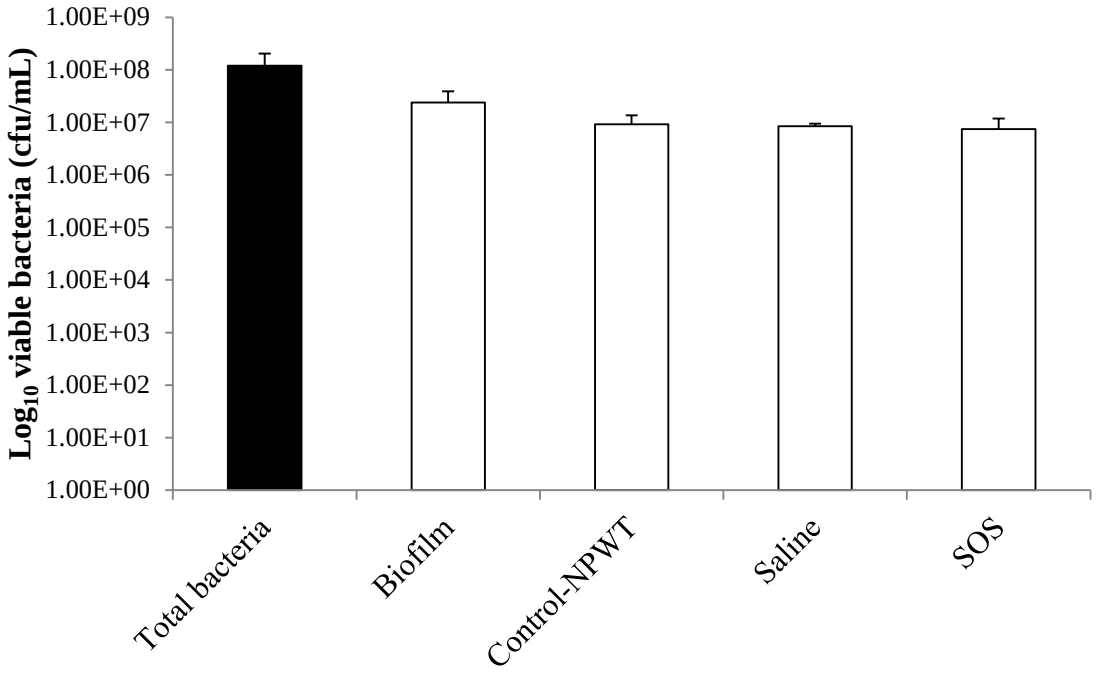
Antimicrobial efficacy of SOS against mature biofilms in an ex vivo porcine skin explant model

Levels of *P. aeruginosa* PA01 viable bacteria after twelve cycles of negative pressure therapy and instillation of saline or SOS are shown in Figure 6.7. The total bacterial bioburden (planktonic + biofilm) growing on the porcine skin explant was 8.0 log10 cfu/mL, of which 7.1 log10 cfu/mL were biofilm bacteria, as defined by being tolerate to incubation in 50x MIC gentamicin for 24 hours at 37°C.

When porcine skin explants with mature *P. aeruginosa* biofilm were exposed to 12 cycles of negative pressure wound therapy alone without instillation of any solution (Control- negative pressure wound therapy), which is equivalent to "pulsed or intermittent negative pressure wound therapy", the level of PAO1 cfu was reduced to 6.9 log10 cfu/mL of total PAO1 bacteria. When the porcine skin explants were treated with negative pressure wound therapy with 12 cycles of instillation with saline with a 10-minute exposure time, the level of PAO1 bacteria was the same with 6.9 log10 cfu/mL.

Changing the instillation solution to SOS and using the same 12 cycles of instillation, the level of PAO1 bacteria was essentially the same as saline instillation, with 6.8 log10 cfu/mL surviving the instillation treatment. In contrast, planktonic and biofilm bacteria were completely eradicated using the *in vitro* CDC biofilm reactor laboratory test.

Figure 6.7 10^8 cfu of P. aeruginosa PA01 inoculated onto porcine skin explants. After 3 days of growth at 37˚C, the average cfu of viable total bacteria or biofilm bacteria pre-and-post 12 cycles of 10-minute instillations are reported. Instillation was undertaken with saline or SOS solutions or only negative pressure wound therapy without instillation.



As shown in figures 6.8 and 6.9, SEM of the wound area in the porcine skin explants post-treatment with SOS after 12 cycles of 10-minute exposure demonstrated very thick continuous biofilm on untreated explants.

Figure 6.8 Un-treated pig skin showing Bacterial shape of Gram-negative rods – P. aeruginosa. Arrows illustrate microbial aggregates/bacteria.

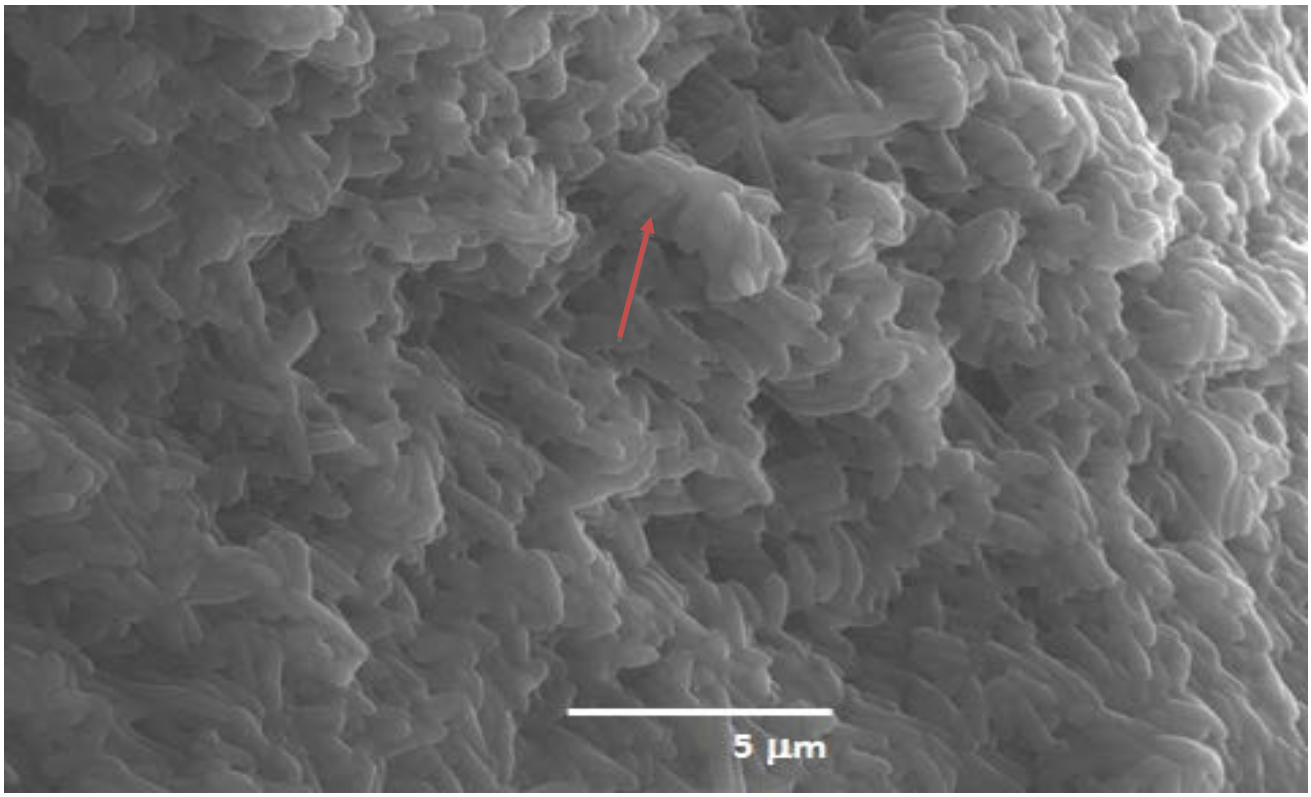
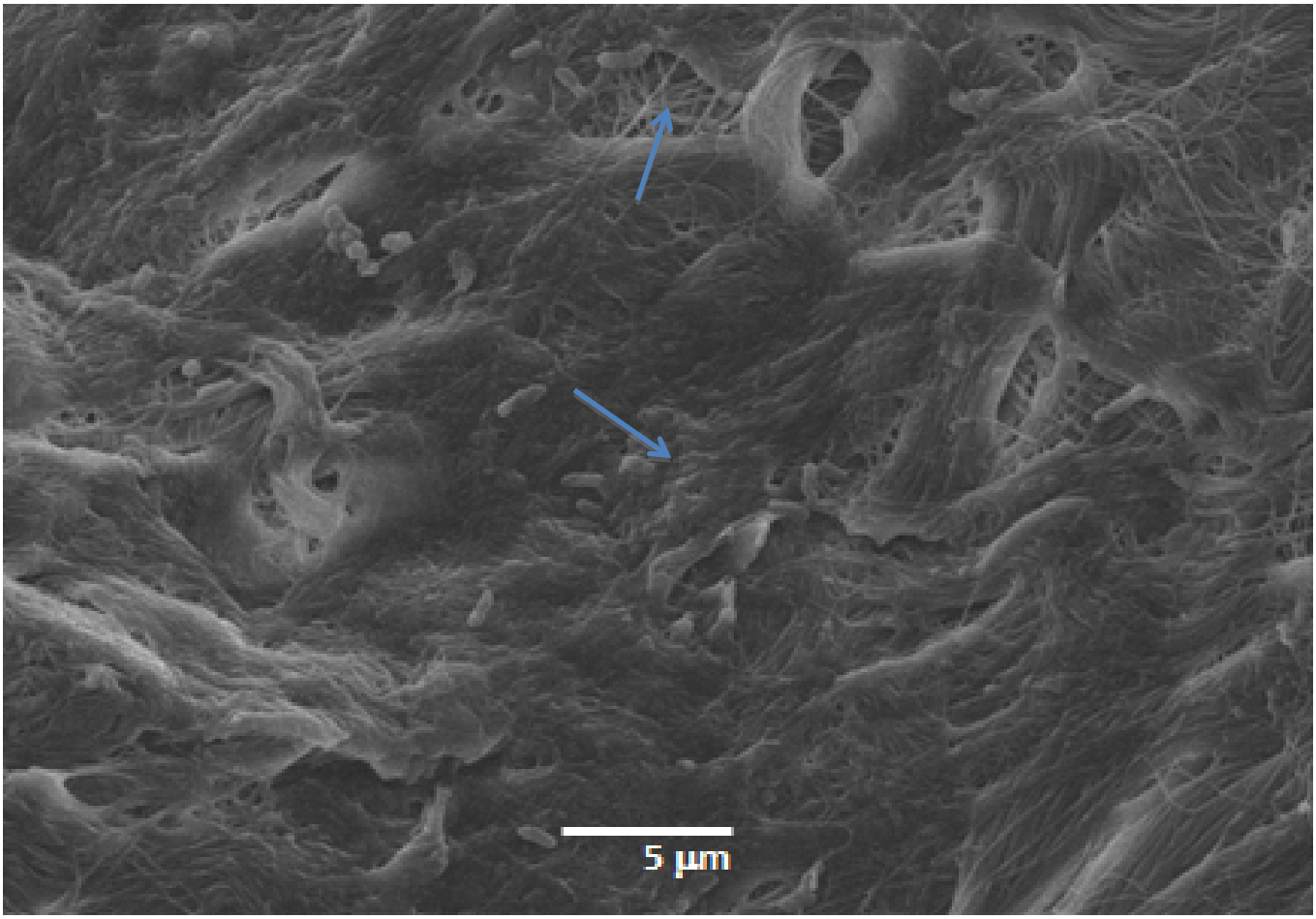


Figure 6.9 Explants treated with SOS instillation also showed reduction of biofilm structure but a persistence of attached bacteria. Thick extracelluar matrix creating a stringy web like apperance. Some EPS has collpased as part of the dehydration process of SEM.



The effect of SBMO against the microbial load and diversity of DFUs complicated by biofilm in vivo

A total of 10 patients with chronic non-healing DFUs were enrolled. 1,306,086 high quality DNA sequences were generated (before = 623,117, after = 682,969), with a median of 61,132 per sample level data (Range = 5,702 to 168,421). The clustering of operational taxonomic units (OTUs) identified 1,976 unique taxa of which low abundance OTUs were removed (<0.1%), leaving 124 OTUs for further analysis.

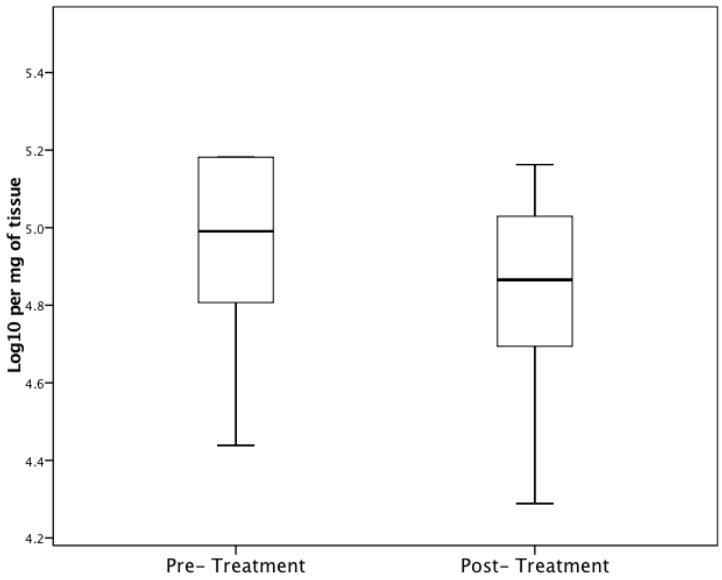
Confirmation of the presence or absence of biofilms in each DFU

Biofilms were visualized and confirmed in all 10 participants using SEM. Biofilm architecture was graded using an arbitrary sliding scale from a score of 5 (heavy biofilm) to 0 (no biofilm) as previously reported [78]. The median value of DFU biofilm architecture reduced from pre-treatment 4 (large micro-colonies ~100 cells, and a continuous film/matrix) to post-treatment 3 (large micro-colonies ~100 cells).

Microbial load of chronic non-healing DFUs complicated by biofilm

The application of SBMO for 15-minutes exposure daily, for seven days, resulted in no change to the total microbial load (Figure 6.10) (median log10 microbial load pre-treatment = 4.9 log10 16S rDNA copies / per mg of tissue, versus 4.8 log10 16S rDNA copies / per mg of tissue (p = .43).

Figure 6.10 Effects of SBMO pre-and post-treatment of ten chronic non-healing DFUs. Box-and-whisper plot represents the median log10 16S rDNA copies / per mg of tissue values of all 10 patients.

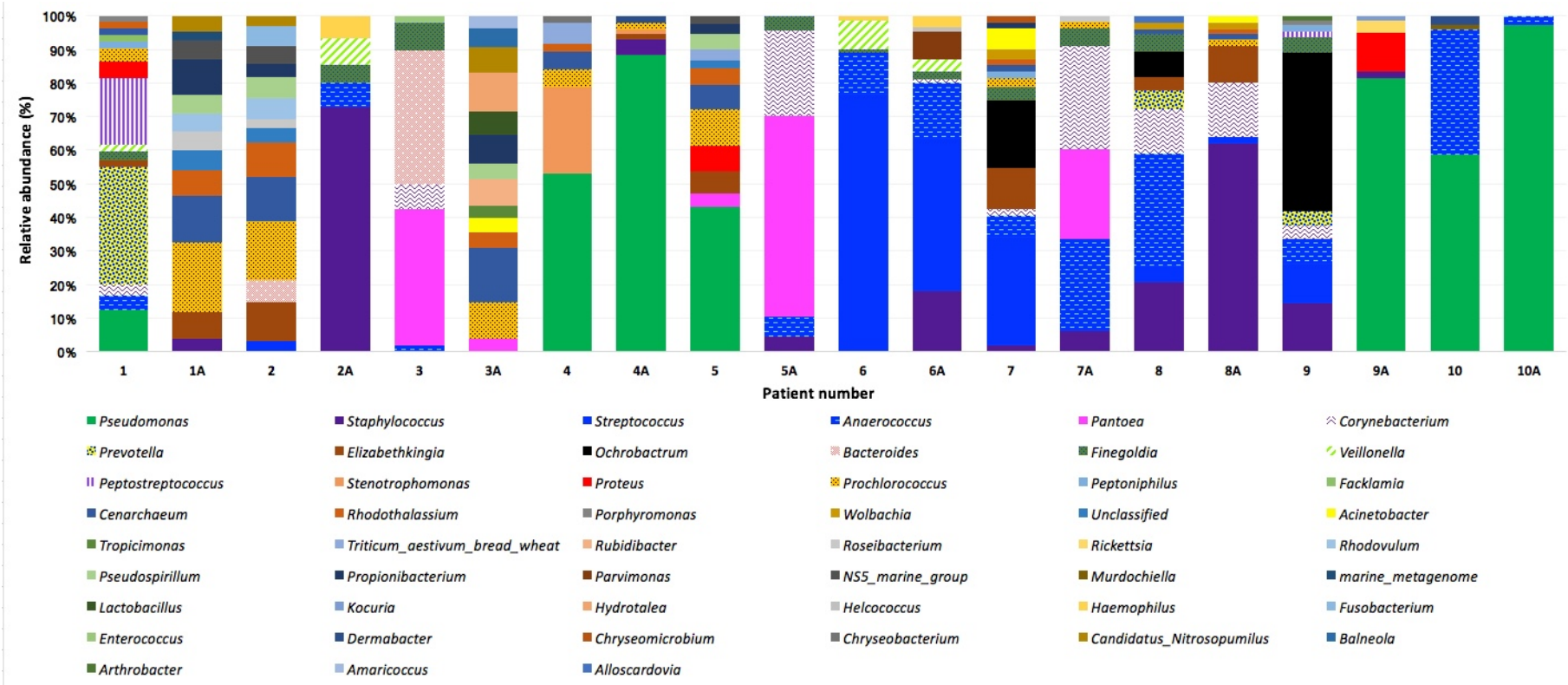


Analysis of community richness and diversity of chronic non-healing DFUs treated with SBMO.

The most abundant OTUs contributing to >1% of the microorganisms within individual DFUs are noted in Figure 6.11; *P. aeruginosa*, *S. aureus*, *Anaerococcus* spp., *Prevotella* spp., and *Streptococcus* spp. were most commonly identified.

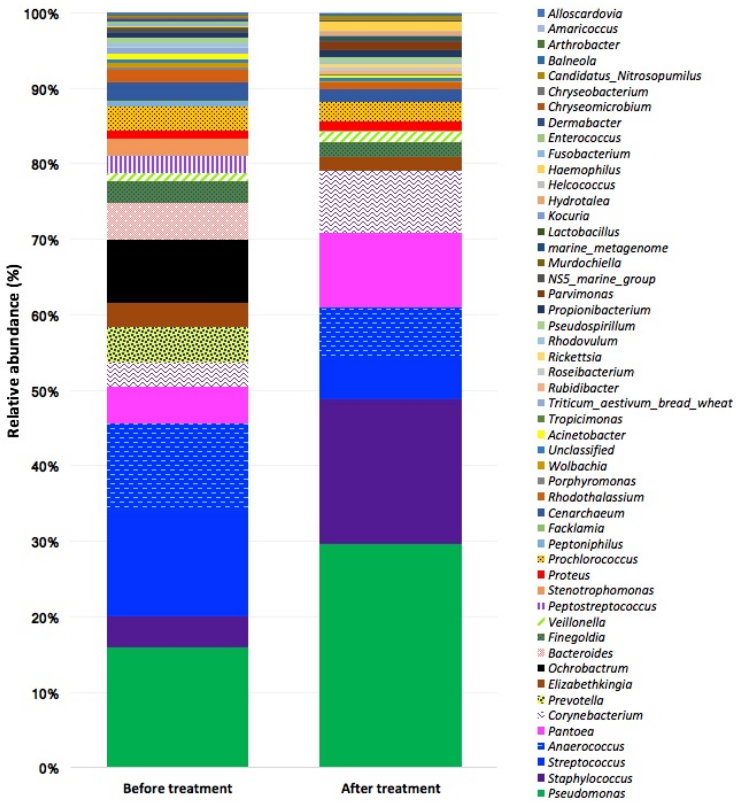
Figure 6.11 Effects to microbial communities following treatment with SBMO. Pairwise comparisons of pre-and post-treatment microbial communities at the genus level for individual wound data identifying the relative abundance (%) of OTUs.

(a)



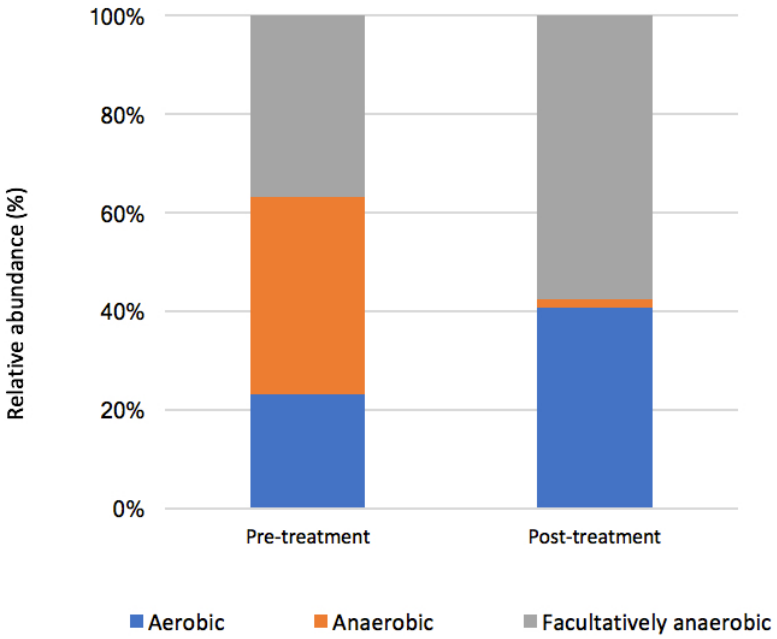
The % relative abundance of OTUs. *P. aeruginosa* and *S. aureus* increased in all but one patient post-treatment with SBMO (Figure 6.12), with pooled data from all samples identifying this to be statistically significant for *Staphylococcus* spp., DNA copies (p = .04).

Figure 6.12 Pooled analysis from all samples identifying overall relative abundance pre-and-post-treatment with SBMO.



Additionally, the number of both *P. aeruginosa* and *S. aureus* relative abundance increased in all but one patient following treatment with SBMO. Only patient 9 seemed to experience a reduction to *S. aureus* levels, however a significant increase in *P. aeruginosa* was noted as a result. SBMO reduced the total number of anaerobic microorganisms (Figure 6.13), but a concomitant increase in both aerobes and facultative microorganisms was noted.

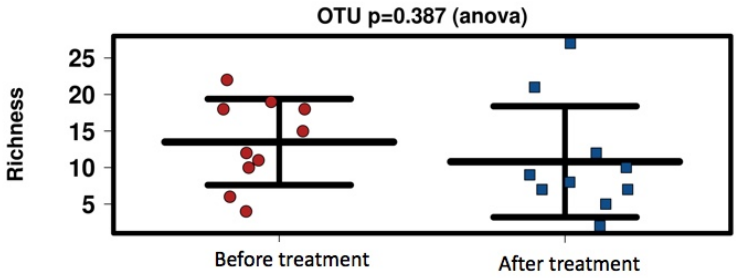
Figure 6.13 Relative abundance (%) normalised to microbial load for each individual wound and then represented as pooled data with regards to aerotolerance.



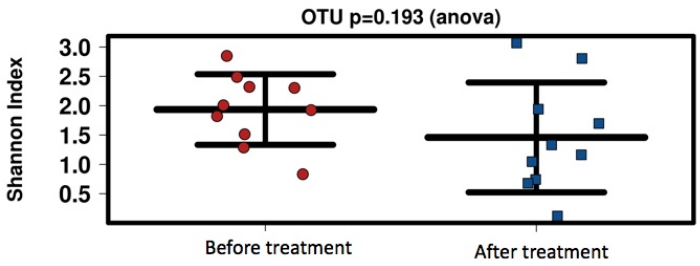
Analysis of microorganisms contributing to >1% of microbial communities in individual DFUs and from pooled data were analysed from alpha and beta diversity measures. Chronic DFUs prior to treatment were rich and diverse, yet there were minimal changes to community richness (p = .3), diversity (p = .1) or community composition of DFUs post-treatment (p = .9) (Figure 6.14 a-c).

Figure 6.14 Analysis of microbial communities alpha and beta diversity. (a) Richness index identifying unique OTUs. (b) Shannon Index identifies number of OTUs and their evenness. (c) Bray-Curtis dissimilarity matrix with PERMANOVA identifies differences (or not) in community composition.

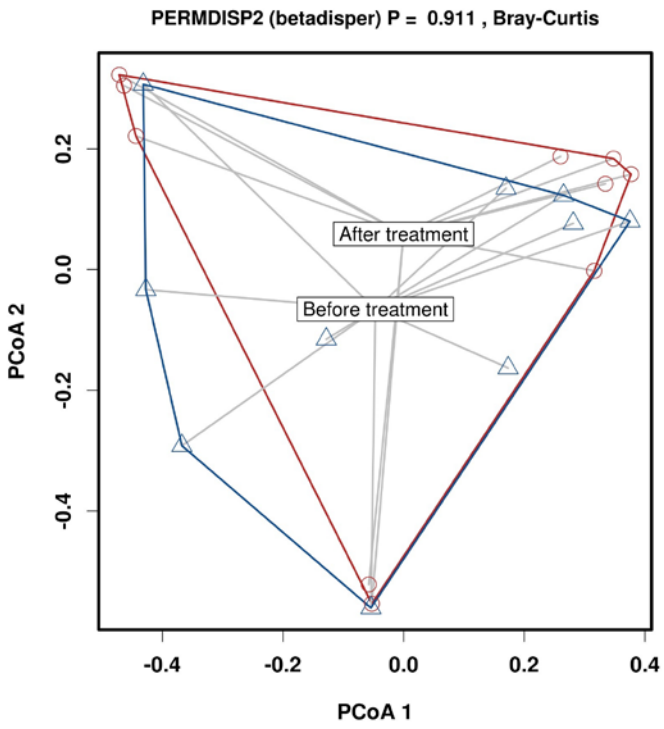
(a)



(b)



(c)



6.1.4. DISCUSSION

Summary of key findings

We systematically tested the performance of topical antimicrobial solutions using short exposure times for *in vitro* and *ex vivo* models and an *in vivo* human trial. Our results suggest that the performance of these solutions is poor when challenged against mature biofilms using short exposure times that mimic real clinical use (i.e. 15-minute application). Clinicians using topical antimicrobials to eradicate bacteria in chronic wounds as a single therapy under the assumption of removing biofilm may experience poor clinical outcomes. Clinicians should consider multi-faceted strategies that include sharp debridement as standard [309,370].

What this study adds to the available evidence and new recommendations

There are many facets to the management of chronic wounds, with a large focus on managing wounds colonised or infected with either planktonic or biofilm phenotype microorganisms. Whilst there is a plethora of data pertaining to the effectiveness of topical antimicrobials *in vitro* against both planktonic and sessile microorganisms, here we identify the inherent limitations of *in vitro* methodologies that fail to consider clinically relevant biofilm models when testing topical antimicrobials for use in wound care [288,301,365].

In particular, *in vitro* models testing topical antimicrobial wound solutions have not considered the clinical applications of the products use with regards to the time of exposure [249], and outcomes are often reported after 24-hour exposure times. This does not reflect the typical clinical pattern of usage of these products or the 'instructions for use' explained in product inserts. Nor does the use of immature biofilms (early forming biofilm 24 hours old) that have a less organised structure, a more active metabolism and a less pronounced stress response,

truly depict the complex, mature and highly tolerant biofilms identified in many chronic wounds [90,371].

This may explain why some of the topical antimicrobials tested at clinically relevant times in this study performed poorly. Our *in vitro* model utilised two clinically relevant bacteria, *P. aeruginosa* and *S. aureus*, which have been noted as causes of delayed wound healing and as pathogens of infection [141,372]. Testing the efficacy of solutions over a single 15-minute exposure time *in vitro*, we identified great variability in test performances. In general, surfactant based topical antimicrobials performed poorly (except for SBPHMB against *P. aeruginosa*) and was no more effective than normal saline (non-antimicrobial).

Traditional antiseptics such as CHX and PVP-I were highly effective, whilst new generation solutions such as SOS were also highly efficacious. Chlorohexidine is a cationic bisbiguanide with a broad-spectrum biocide active against both Gram-positive and Gram-negative bacteria. [373] Its primary action is against the negatively charged bacterial cell wall, leading to increased cell permeability resulting in cell death [373].

The efficacy of Chlorhexidine in reducing or eradicating single or multi-species biofilm has been demonstrated *in vitro* [374-376], with the combination of cetrimide to chlorhexidine producing enhanced antimicrobial or anti-biofilm activity. One explanation for the effectiveness of CHX *in vitro* in this study may be explained by the cationic surfactant properties of cetrimide that has a demonstrated capacity to decrease the mechanical stability of biofilm (in addition to its proven bactericidal activity), but further work is required to elucidate these effects in wound models [377].

PVP-I also performed well *in vitro*, and as a broad-spectrum microbiocide is capable of inactivating Gram-positive and Gram-negative bacterial species, bacterial spores, fungi, protozoa and several viruses [378]. PVP enables the delivery of free iodine to a target cell membrane, where its mechanism of action is to destabilise the structural components of cell membranes [378]. It has demonstrated activity against biofilms *in vitro* [379,380]. More recent *in vitro* data on the performance of a wound-care related PVP-I on multi-species biofilms using the CDC reactor have corroborated this study's results [299].

More recently, "newer generation" topical solutions with antimicrobial properties such as superoxidized solutions have been utilized as anti-biofilm therapies in wound-care, even in the presence of a low evidence base. SOS contains the primary ingredient of hypochlorous acid (which is not new generation), and only one *in vitro* study is available that used the concentrations of SOS found in current in wound-care solution [301]. Using a continuous flow tube reactor (to mimic the clinical scenario of a catheter) to grow mature six-day old *P. aeruginosa PA01* biofilms, Sauer and colleagues utilised SOS at the same concentration (80 ppm) reported in this study, to achieve a 2.5 log10 reduction after 60 minutes exposure.

Our study identified that SOS could eradicate *P. aeruginosa* biofilms in addition to performing well against *S. aureus* biofilm. This contrasted with the porcine skin model, where SOS achieved only 0.77-log10 reduction against *P. aeruginosa PA01* biofilms. Potential explanations to describe these results could be attributed to the two different strains of *P. aeruginosa* that were used for the study. The *in vitro* model utilised *P. aeruginosa* (ATCC® 25619™) and the porcine skin explant utilised *P. aeruginosa (*PA01, ATCC® BAA-47™). Sauer and colleagues also utilised *P. aeruginosa (*PA01, ATCC® BAA-47™). Interestingly, the use of *P. aeruginosa PA01* strain yielded results that identified a reduced effectiveness of

SOS. It is possible that whilst our *in vitro P. aeruginosa* (ATCC® 25619™) strain readily formed biofilm with the characteristic *P. aeruginosa* architecture, it did not develop a high-level biofilm-specific resistance [381], that may have arisen in the PA01 strain.

The different results observed for SOS *in vitro* versus the porcine skin model may be explained by the surface the biofilms were formed on (i.e. the soft tissue dermal matrix of porcine skin which more closely represent an actual wound bed compared to an abiotic polycarbonate disc). This may have attributed to alterations in microbial behaviour in response to the presence of biotic signals or organic material [382,383].

Biofilms grown on biotic substrates or *in vivo* often do not display the morphological or architectural characteristics of those grown *in vitro* (e.g., mushroom structures and towers), which are important parameters that undoubtedly affect bacterial behaviour [384]. Lastly unlike an abiotic surface, porcine skin has a striking similarity to human skin in terms of its structure and this is important given that microbial aggregates have been identified as forming not only on a wound surface, but also penetrating to deeper structures in a non-random distribution [41]. In this scenario, any topical solution applied to a contact surface would have to penetrate a biofilm formed on that contact surface in addition to then penetrating between tissue cells. This presents a greater challenge than that already posed by biofilm tolerance mechanisms and may contribute to the reduced effectiveness of topical antimicrobials.

Lastly, the performance of SBMO was tested on human tissue in an *in vivo* study on chronic non-healing DFUs. SBMO was applied daily for 15 minutes over a seven-day treatment period, with the results identifying no change in the total microbial load from tissue biopsies.

Interestingly, our *in vivo* results identified a correlation between the poor performance of SBMO against *P. aeruginosa* and *S. aureus* that was also seen *in vitro*.

16S rDNA next generation sequencing was performed to understand the effects of SBMO on microbial communities in chronic non-healing DFUs. The relative abundances of both *P. aeruginosa* and *S. aureus* within most DFUs increased post-treatment. Conversely, an overall reduction in the relative abundance of anaerobic microorganisms and low frequency taxa (microorganisms contributing <1% relative abundance) was noted, but the total microbial loads within these wounds did not decrease. This suggests that more dominant species such as *Staphylococcus* spp. or *Pseudomonas* spp. benefit from the increased nutrient availability caused by disruption to the microbial community that resulted through removal of competing microorganisms [14] thus sustaining the microbial load within tissues.

Treatment with SBMO resulted in the reduction in relative abundance of anaerobic microorganisms. Anaerobic microorganisms have been identified as part of polymicrobial communities cited for their involvement as a cause of delayed wound healing [6,385], as pathogens of infection in the diabetic foot [47] and in biofilm production [386]. In this instance, reducing their numbers would seem like a positive step to reducing microorganisms with the potential to negatively impact the wound environment. Unfortunately, this might not be true given the concomitant increases in *Staphylococcus* spp., and *P. aeruginosa* that are equally capable pathogens.

To assess the overall effects of SBMO treatment on DFU microbiota (community richness, diversity, structure and composition), DNA sequence data were analysed using QIIME [98].

Minimal reductions were seen in the number of OTUs (Richness) and community diversity of chronic DFUs post-treatment. In a recent study by Loesche *et al* (2017) [31], the temporal analysis of chronic DFUs showed that patient samples that had received systemic antimicrobial therapy had no alterations to species richness or diversity, and that antimicrobial exposure did not drive microbiota variation. Instead the data indicated that antimicrobial exposure disrupted the microbiota when antimicrobials were specifically directed to treat underlying wound infection.

We find a similar pattern of events with our data, in that exposure to SBMO had some effects when we explore our samples individually. For example, sample 2 experienced a significant disruption to its microbiota. Pre-treatment *Staphylococcus* spp. contributed <1% relative abundance, and post-treatment this significantly increased to >65%. Similar patterns are seen across our data, but it is not possible to infer if these changes would result in positive or negative effects on a wound. This intriguing aspect requires further longitudinal sampling that maps microbiota disruption to wound outcomes.

Our molecular-based data using16S rDNA, while informative in describing "who is there", is unable to truly define "who is doing what" [325]. In some wounds where anaerobic microorganisms are acting synergistically with aerobic counterparts to increase pathogenicity or virulence in a chronic wound, their reduction may likely lead to positive effects. Conversely, and providing food for thought, any perturbations to the complex microflora seen within chronic wounds may lead to microbial dysbiosis. Of particular significance is the reduction in microbial diversity, which may directly contribute to pathogen selection and persistence. [15] Longitudinal studies are required to determine if alterations to the microbial diversity of chronic non-infected wounds seen through using topical antimicrobials leads to future complications.

Limitations

The CDC biofilm reactor used *in vitro* was performed under flow allowing mature biofilms to form on the polycarbonate coupons. This abiotic surface does not reflect the complexity of human tissue and the host immune response. Secondly, most chronic wounds are contaminated with multiple species of bacteria [90] and this study utilised single species biofilms *in vitro.* That aside, our model tested clinically relevant exposure times against clinically relevant microorganisms involved in both chronic and infected wound types in screening the performance of topical antimicrobial solutions.

qPCR was utilized to measure total microbial load *in vivo* [15] however this method has limitations in its inability to differentiate live or dead bacteria. The log reductions noted in this study therefore represent the minimal response and we acknowledge that some of the bacteria detected by qPCR could be dead, resulting in a lower calculable efficacy.

Overall, the limitations *in vitro* were circumvented by the addition of an *in vivo* study. The cost of performing this study was a factor in not being able to test a wider range of topical antimicrobials *in vivo.* Further studies incorporating a human *in vivo* design may be required to understand the efficacy of products tested in the *in vitro* stage of this study against microbial biofilms. However, when considering the group of studies we performed collectively, there is a strong correlation between exposure time and efficacy.

Summary

Polymicrobial communities forming biofilms in chronic wounds may have extended time periods to develop complex, highly tolerant communities that differ greatly from single species biofilm models grown on glass coupons for 24-72 hours. The discrepancies between the three

different test parameters in this study raise an important question over *in vitro* testing for anti-biofilm therapeutics, where results identifying potential effectiveness against biofilm, differ markedly when the test parameters are changed.

In vitro testing for anti-biofilm strategies are a screening tool identifying potential therapeutics that may perform well on the next stage of testing (i.e. when taken to animal models or to clinical studies). The effectiveness of an anti-biofilm therapeutic at this *in vitro* stage is not absolute, yet for many medical devices companies this is the only data available for use in the promotion of products. When using porcine explants and human *in vivo* tissue samples, our data is highly suggestive that the exposure time of topical antimicrobial wound solutions and irrigation solutions is too short, and that exposure time is critical in determining the efficacy of these products. Clinicians using these topical antimicrobial solutions as a sole therapy under the assumption of killing or eradicating biofilm should consider adopting multi-faceted strategies that include sharp debridement as gold standard.

CHAPTER 7

7.1 APPROACHES TO BIOFILM-ASSOCIATED INFECTIONS: THE NEED FOR STANDARDIZED AND RELEVANT BIOFILM METHODS FOR CLINICAL APPLICATIONS

(APPENDIX 12) **MALONE, M.,** GOERES, D., GOSBELL, I., VICKERY, K., JENSEN, SO., STOODLEY, P. APPROACHES TO BIOFILM-ASSOCIATED INFECTIONS: THE NEED FOR STANDARDIZED AND RELEVANT BIOFILM METHODS FOR CLINICAL APPLICATIONS. EXPERT REVIEW OF ANTI-INFECTIVE THERAPY 2017. (15); 2, 147-156.

Candidate contribution:

- ✓ Worked as primary author for publication.
- ✓ Wrote the manuscript in full.
- ✓ Incorporated feedback and editions from co-authors
- ✓ Submitted the manuscript as primary author.

7.1.2. INTRODUCTION

Since the early 1970's, an explosion of research on the concept of biofilms and their involvement in human health and disease have appeared in the medical literature [131]. This new wealth of information, driven largely by advancements in emerging technologies and techniques applicable to the study of bacterial populations in situ, have advanced the understanding of "microbial biofilms". The concept of biofilms in human health and disease is now universally accepted in chronic wounds [12,141] periodontal disease and dental caries [134,387], cystic fibrosis [135,136,388], in-dwelling medical device infection [389,390], otitis media and other upper respiratory infections [139,140], orthopaedic infections [391] and tuberculosis [133].

Current definitions have described biofilms as microbes attached to surfaces or to each other in aggregates or clumps. They encapsulate in a self-produced extracellular polymeric substance (EPS) or matrix, that can also contain host derived components. As such biofilms show extreme tolerance to antimicrobials and host defenses [196,392-394]. A plethora of *in vitro* biofilm models have elucidated that bacterial biofilms are more tolerant to antiseptics and disinfectants [395] as well as withstanding antimicrobial concentrations 100 to 1000 times higher than that of planktonic counterparts [145,172]

Despite the wealth of research undertaken to identify biofilm tolerance to antimicrobials, no single causative mechanism has been identified. Instead it has been suggested that a likely combination of factors contributes to biofilm tolerance [265,278] with several areas of interest including but not limited to; slow or incomplete permeation of antimicrobials through extracellular polymeric substance (EPS) [172,396], altered microenvironment and niches within

biofilms promoting slow growth rates and adaptive stress response [129,177], efflux pumps [276], and the role of low frequency dormant "persister" cells [257].

Regardless of whether researchers fully uncover the answers to the biofilm riddle of tolerance, the practical implications are that individual patients suffer with prolonged chronic infections that often require multiple rounds of antibiotics [145]. The current treatment strategy for chronic infections comes at a high cost to the healthcare system and, more importantly, to the patient, both economically and in the potential loss in their quality of life.

7.1.3. EXPLORING THE CONCEPT OF WHAT IS A RELEVANT REDUCTION FOR MEDICALLY RELEVANT BIOFILMS?

Antimicrobial therapies for acute infections based on minimum inhibitory concentrations (MIC) (planktonic microorganism's susceptibility to antibiotics) target rapidly multiplying planktonic microorganisms with high efficacy. Therapies based on MIC results employed against biofilm phenotype microorganisms that differ markedly in both their physiology and activity typically fail to eradicate the problem, leading to a chronic infection for the patient. For some patients, with in-dwelling medical devices for example, who have failed anti-biofilm strategies, the infection cannot be resolved until the material is completely removed [397].

Researchers generally evaluate the efficacy of anti-biofilm strategies using susceptibility test results based on assays that identify the minimum biofilm eradication concentration (MBEC assay) through *in vitro* models such as the Calgary biofilm device [398]. The lack of correlation between conventional susceptibility test results and therapeutic success in chronic infections may be reflective of biofilm presence. A recent Cochrane review on standard versus biofilm

antimicrobial susceptibility testing to guide antibiotic therapy in cystic fibrosis identified that biofilm susceptibility testing was not superior to conventional antimicrobial susceptibility testing[399]. The Cochrane review suggests that biofilm antimicrobial susceptibility testing may be more appropriate in the development of newer, more effective formulations of drugs that can be tested in clinical trials.

In addition to antibiotics, various agents have been explored for anti-biofilm activity. These have included peptides, antiseptics and oral and topical antimicrobials. How these agents are delivered to the biofilm has also varied greatly with mechanisms including coatings, drug eluting materials, wound gels, nanoparticles, irrigations and solutions, all being explored. Several alternate techniques have been developed to quantify outcome measures for these agents *in vitro.* Biofilm biomass has been explored, typically in 96-well microtiter plates and flow systems using staining methods (crystal violet, Syto9 staining) with optical density (OD_{nm}) or confocal laser scanning microscopy (CLSM) to detect live/dead cells (expressed as percentages or OD_{nm}) [400,401]. Plate counts to enumerate viable cells that calculate antimicrobial efficacy expressed as cfu/ml, cfu/surface area, cfu/per mg tissue have also been utilised.

The absence of a "target" reference value required to ascertain the "effectiveness" of anti-biofilm strategies in aiding the host immune response to clear infective microorganisms is a problem. This has important consequences at a treatment level where clinicians often seek guidance from laboratory-based studies (often due the lack of *in vivo* data) to choose the most relevant and effective agent to reduce microbial colonization/infection. These decisions have historically been based on the use of planktonic paradigms.

Importantly, when deciphering what may be a “target reference” there are two sides of the fence to consider. Firstly there is a regulatory perspective that looks to determine a “target reference” based on standardized approaches using statistical attributes of an agent [402,403]. Secondly, there is the issue of how well *in vitro* or *ex vivo* results translate to clinical efficacy and if those “target references” correlate to improvements in clinical symptoms and resolution of chronic infections.

With respect to what would be a potential “target reference” value, there are no data to support what a reasonable figure would be. A target reference value may move depending on the type of infection, the infecting strain or the immune status of the patient. For example, data to support a reasonable target reference value for *in vitro* testing must take into consideration that when bacteria are expressing biofilm phenotype, infectivity *in vivo* may alter drastically. The most conservative approach is that the drug or device must demonstrate complete eradication of the biofilm in *in vitro* testing.” The obvious approach to determine a reference value would be to transition from *in vitro* testing to *in vivo* clinical trials, as this would allow direct observations of what worked and what did not. In many cases, however, it may not be possible to obtain biofilm data directly from patients.

The United States Environmental Protection Agency (EPA) addressed this concern for human health biofilm disinfection claims by proposing a 6-log reduction in biofilm as a standard. However, if industry wants to be “highly confident that they will achieve this target log reduction, then they need to formulate the biocide to completely kill the biofilm.

7.1.4. HOW SHOULD WE APPROACH ASSESSING THE "EFFECTIVENESS" OF ANTI-BIOFILM THERAPIES BASED ON *IN VITRO* MODELS TO PREDICT CLINICAL RESULTS

Biofilms exist in many niches and vary significantly from niche to niche. This likely restricts the ability to develop an assay that could be used universally, especially also given the large variation in biofilm architecture from *in vitro* to *in vivo*. Whether one is evaluating biocides for use against biofilm in toilets, or antibiotics to treat chronic wounds, it is virtually impossible to perfectly mimic an actual infection or environment in the laboratory.

Various publications have stated the need for the standardization of methods for assessing the "effectiveness" of anti-biofilm therapies.

A problem however, facing anyone attempting to decipher the literature or attempting to replicate biofilm models for new therapies has been the lack of standardized methods for experimentally studying biofilms. This has caused much confusion when attempting to compare results between different research groups and has led to discrepancies when attempting to replicate the results between different laboratories. The lack of standardised methods also means there is no pathway for companies to follow when attempting to register a new device or drug with a regulatory agency.

In the most applied sense, standard methods development is the creation of laboratory protocols for comparison, both within a single laboratory and among various laboratories. Researchers choose to use a standard method for various reasons. For instance, because every step of the laboratory process is exactly defined, a standard method is useful for teaching proper laboratory protocol or monitoring equipment performance. The impetus for the development of many microbial standard methods, though, is efficacy testing for product registration with a

regulatory agency. Regulatory agencies require efficacy data when a product is registered to ensure the quality, safety, and efficacy of antimicrobials (biocides, disinfectants, sterilants).

For this purpose, standardized methods that are repeatable, reproducible, rugged and responsive are required [404]. A standard method should also be reasonable, meaning it should utilize equipment that is "typical" for a laboratory and it should not require an excessive amount of time, supplies or highly specialized training. Many biofilm research methods can uncover intriguing scientific insights even though the results are qualitative. However, regulatory authorities and standard setting organizations mostly prefer quantitative measures of efficacy.

Uniform test conditions permit comparison of results between products and laboratories. To support a claim of bactericidal action for a disinfectant for example, efficacy against planktonic *P. aeruginosa*, *P. vulgaris*, *E. coli* and *S. aureus* is required by the Australian Therapeutic Goods Administration (TGO 54) [405], with efficacy against similar organisms being required by other regulatory organizations. All these organisms are biofilm producers and are associated with clinically relevant biofilm infections, so it seems reasonable to include anti-biofilm testing for these (or a subset of these) organisms. Unfortunately, standard methods only exist for biofilms formed by *P. aeruginosa* (ASTM Methods for E2196, E2562, E2647, and E2799).

Importantly, a relevant laboratory method should adequately emulate "real use" conditions so that a laboratory test is predictive of how well a device (or test product of interest) will perform *in vivo*. Highlighting the decontamination of equipment and clinical surfaces with disinfectants/ sterilants as an example, it is of importance for users (or clinicians) responsible for the decontamination of instruments or surfaces to understand that the product they are using has been tested under conditions that best resemble the purpose to be applied to, such as the hospital

environment. Therefore, in terms of relevance, there are two basic strategies that researchers should strive to answer and that clinicians should strive to understand.

The first strategy is to engineer a biofilm in a laboratory test to have specific characteristics that emulate the biofilm *in vivo*, matching for example, the architecture, thickness, strength of attachment and host factors such as proteins or immune cells. This is because alterations in any of these parameters can lead to alterations in the test outcomes. For example, the sensitivity of biofilm to disinfectants varies with both the age of the biofilm and the method of growth [406]. This is well demonstrated in a paper by Buckingham-Meyer *et al* (2007) [362] where kill rate (log reduction) decreased as the amount of shear on the test biofilm during growth increased.

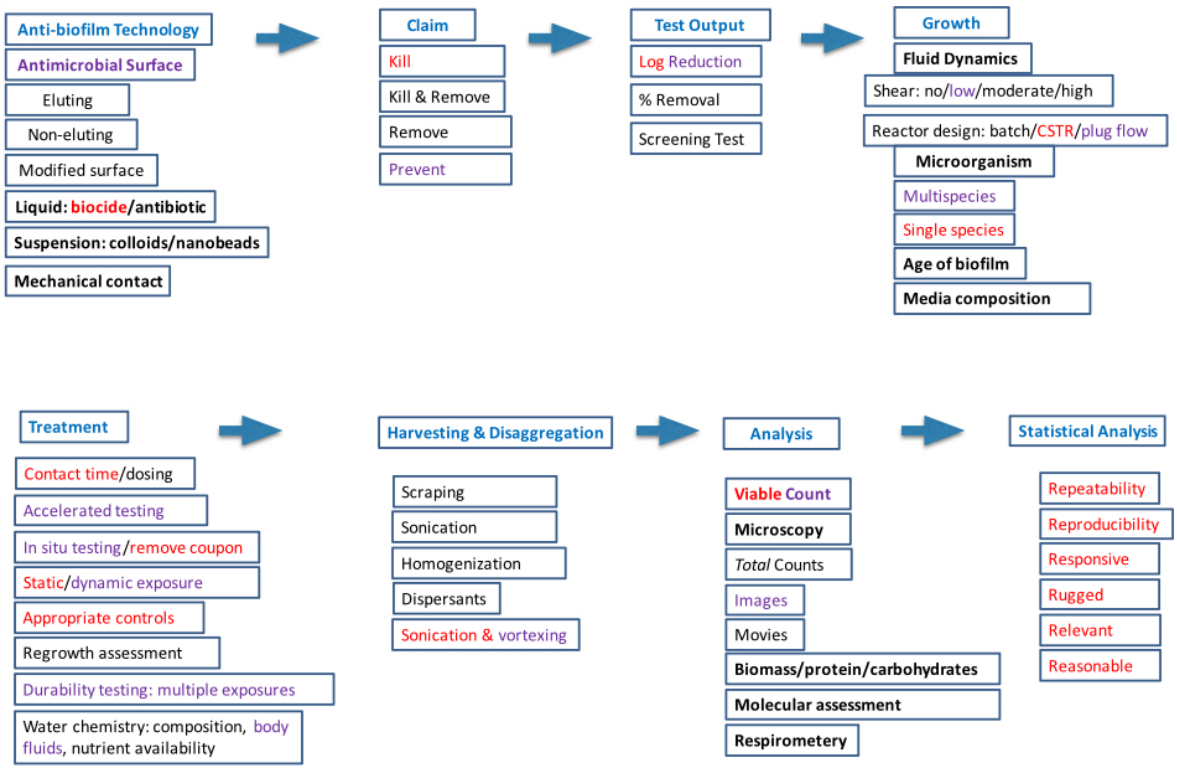
The ASTM standard biofilm methods were developed based upon this relevance strategy (Table 7.1). By employing basic fluid dynamic concepts with regards to fluid shear and flow dynamics, the ASTM methods describe how to grow a biofilm that represents a general biofilm grown under high shear in a continuous stirred tank reactor (CSTR) (ASTM Method E2562) [407], in medium shear in a CSTR (ASTM Method E2196) [408], low shear in a plug flow reactor close to the air liquid interface (ASTM Method E2647) [409] and minimal shear in a batch reactor (ASTM Method E 2799) [410]. Others have recently reviewed the applicability of the biofilm reactors described in the ASTM Methods for various applications [122,411,412].

Biofilm model	Method	Nutrient availability	Potential applications and relevance
Rotating disc reactor (annular reactor) (ASTM E2196 – approved 2002)	This test method is used for growing a reproducible *P. aeruginosa* biofilm in a continuously stirred tank reactor (CSTR) under medium shear conditions.	Open system Dynamic Continuous flow	Rotating disc reactors are designed for laboratory evaluations of biocide efficacy, biofilm removal, and performance of anti-fouling materials. Example is to model a toilet bowl [413]. It is important to note that the rotating disk and CDC reactor were not originally designed to study medically relevant biofilms.
Drip flow reactor (ASTM E2647 – approved 2008)	This test method is to grow, sample, and analyze a *P. aeruginosa* biofilm under low fluid shear and close to the air/liquid interface.	Open system Dynamic Batch or continuous flow	DFR are employed for growing biofilms for direct in situ visualization. The DFR can model environments such as food-processing conveyor belts, catheters, and the oral cavity [414 415].
CDC biofilm reactor (ASTM E2562 – approved 2007)	This test method is used for growing *P. aeruginosa* biofilm under moderate to high shear. The resulting biofilm is representative of generalized situations where biofilm exists under high shear rather than being representative of one environment.	Open system Dynamic Batch or continuous flow	Studies that utilized this reactor showed that it could be used for detecting biofilm formation, characterizing biofilm structure [416] and assessing the effect of antimicrobial agents on the biofilm (Note there is a large body of literature on how researchers are using the CDC, DFR and MBEC for various research applications.)
MBEC assay / microtiter plates. (ASTM E2799 – approved 2011)	This test method specifies the operational parameters required to grow and treat a *P. aeruginosa* biofilm in a high throughput-screening assay.	Closed system Low shear (the reactor sits on a shaker) Batch	MBEC assay allow rapid throughput of multiple samples of anti-biofilm therapeutics such as antibiotics, antiseptics, compounds and peptides [417].
Single tube disinfection (ASTM 2871- approved 2013)	Standard test method for evaluating disinfectant efficacy against *P. aeruginosa* biofilm grown in the CDC biofilm reactor using the single tube method.	The single tube method is only an efficacy test. Biocides are tested in a batch system, with no mixing at room temperature.	This test was originally designed to determine the efficacy of liquid biocides against biofilm (bleach, quats, hydrogen peroxide blends, etc). Although it has been optimized using biofilm grown in the CDC reactor, the original intent was that the biofilm could originate from any biofilm reactor, if the appropriate controls were carried along.

Table 7.1 Commonly employed laboratory models for biofilm investigation.Commonly

The second basic strategy in methods development involves using reactors that incorporate the most important physiochemical and biological characteristics in the environment of interest [418,419]. An effective strategy that was followed for the development of the ASTM biofilm methods was to partition methods into sets of components. For testing the efficacy of disinfectants or antibiotics these components include growing a repeatable and relevant biofilm, applying the antimicrobial treatment, harvesting a sample of the treated biofilm, and analyzing the sample for viable cells. To better visualize this concept, Figure 7.1 shows a product testing and development guidance tree that outlines some of the numerous parameters under consideration for medically relevant biofilm standard methods.

Figure 7.1 Decision tree for product testing and development guidance



The decision-making process for Figure 7.1, begins with understanding the mechanism of action (MOA) of the of the anti-biofilm technology. The technology then determines the regulatory claim. For instance, an antimicrobial surface would most likely be associated with a “prevents initial attachment” or “reduces biofilm accumulation” claim, whereas a biocide manufacturer would most likely pursue a “kills” or “removes” biofilm claim. The claim then determines the necessary test output that will provide the necessary data to support the claim. For instance, a test that measures the log reduction in viable biofilm bacteria would provide the relevant data for “kill” claim. The next step is to determine which laboratory growth and treatment methods best mimic the real-world application.

Various parameters of concern for biofilm methods are included in this figure, but it is important to note that the list is not exhaustive. The growth and treatment will often determine how the laboratory biofilm will be harvested and analysed. For instance, biofilm grown in microtiter plates is often not harvested, but stained directly and placed into a plate reader. Finally, every standard method must meet the statistical attributes listed in the Figure 7.1. The text highlighted in red demonstrates the standardization path taken to measure the efficacy (kill) of biocides against biofilm.

In this case, a single species biofilm is grown under high shear in the CDC reactor. The mature biofilm is removed from the reactor and tested under static conditions for a contact time specified by the biocide manufacture. Appropriate controls are always included. Sonication and vortexing is used to harvest the biofilm and the viable cells are enumerated using viable cells counts. Finally, the proposed method has to undergone a collaborative study to verify that it meets the required statistical attributes. The text highlighted in purple demonstrates a potential strategy for testing antimicrobial surfaces engineered to prevent biofilm attachment.

Despite the above, many researchers involved in biocide disinfection of a surface (not for medical devices or antimicrobial therapies) may still pose questions such as how well does the hydrated biofilm formed on a coupon or in the MBEC plate represent biofilm on a clinical surface which is in a semi-dehydrated state and encased in thickened EPS. It is unlikely that a biofilm formed on coupons or in an MBEC device will present the same challenge to biocides as biofilm that has been subjected to multiple rounds of decontamination e.g. biofilm contaminating endoscope channels [420].

Biofilms form on all material types within the clinical environment, ranging from fabrics to plastics to stainless steel. Therefore, should research design questions be directed towards testing on different types of surfaces? For example, how relevant is a hard surface test as seen with current standards to killing biofilm on fabric? The CDC biofilm reactor (used in ASTM E2562) [407] uses removable coupons and thus has the capacity to compare different hard surface carriers with a range in free energy values and hydrophobicity e.g. glass, plastic, porcelain and steel. The premise of pushing the boundaries of any test condition and allowing researchers the "artistic" flexibility to mimic "real use" conditions often increases the complexity of the test methodology. Typically, methods that try to exactly match every parameter of interest in this manner are complex and therefore, when the method is verified in an inter-laboratory study (or ring trial), they do not perform well.

7.2.4. EXPERT COMMENTARY

Biofilm research has grown exponentially over the last two decades yet there are minimal data correlating *in vitro* results to clinical outcomes. In addition, whilst the medical community has a greater awareness of the role of biofilms in human health and disease, there are still many areas of confusion for clinicians, who in particular find it difficult to understand how *in vitro* methods translate to something of clinical relevance [125]. This begs the question, why are we not further along in the battle against biofilm associated infections? What is holding us back?

In trying to understand why the pursuit of new anti-biofilm therapies has been lethargic in some areas of medically relevant biofilm research, potential explanations are a lack of standardized methods for testing anti-biofilm models that are clinically applicable (to be discussed in the next section) [362,421] , the lack of regulatory guidance for setting performance standards for biofilm related product claims in the medical device arena, a poor understanding of what defines "effectiveness" when applied to anti-biofilm strategies, the slow response of industry in pursuing new anti-biofilm therapeutics, perhaps due to the lack of regulatory guidance, standard methods, the cost of research and development and the cost of appropriate human clinical trials, factors that inadvertently force the industry to test their potential anti-biofilm therapies using methods that do not correlate to clinical outcomes, lack of funding resources to support the development of standard methods and lastly, the slow progression in translating anti-biofilm research and therapeutics to clinically relevant information [122].

With increasing evidence detailing most aspects of biofilm involvement in human health and disease, clinicians and regulatory agencies have been hesitant to accept and pursue anti-biofilm

treatment strategies. In contrast, the chemical disinfection world for example, has lobbied hard for inclusion of anti-biofilm claims on products and these efforts have led to the development, validation and approval of standard methods for testing of anti-biofilm products. Examples of these are five ASTM standard test methods (E2196, E2562, E2647, E2799 and E2871) (Table 7.1). The culmination of working towards developing standardized approaches that industry can utilise has meant that within the next two years, we may well see products with "kills biofilm" claims.

However, the overall the lack of advancement in anti-biofilm strategies from industry that include medical device and biocide companies, may be explained by their haste to test their current therapeutics. Historically promoted for use against planktonic microorganisms in acute infections, the drive to ascertain if antimicrobials now have an action against biofilms may explain why industry are not moving towards new research and development specifically targeting anti-biofilm strategies. A major contributor for this is most likely the significant investment costs required to develop new therapies utilising evidence from *in vitro* through to *in vivo* trials. The experimental designs in human studies, for example, would likely need to include many patients for a statistically relevant conclusion to be reached.

In tandem with a lack of investment from industry is the challenge to find funding to support the development of standard methods, and the time it takes to develop a standard method. Once a standard operating procedure is written, the method needs to go through rigorous intra-laboratory testing to accumulate sufficient data to demonstrate that the method is repeatable, responsive and rugged. This process may take one to two years, depending upon how compatible for standardization the research methodology is. The method is then taken to a standard setting organization where each step is critically reviewed and discussed, a process

which may also take a few years. Finally, the method goes through a multi-laboratory collaborative study to determine the reproducibility of the method. Assuming the method performs well, the process is complete. But, if the method does not do well, it goes back to a standard setting organization (i.e. ASTM group) and is modified, extending the cost and time associated with standardizing it.

Why haven't clinicians lobbied for the same development of anti-biofilm therapeutics? Or why medical device companies have been slow to pursue new therapeutics. One reason to explain this slow progress is when clinicians come across a new drug and/or device, the regulations on the wording of the claim/documentation is focused on curing or preventing infection. Biofilm does not become part of the discussion.

This may seem to be a case of semantics, but simply not having biofilm be part of the discussion means generally it is not included as part of the clinician's decision making in terms of infection management. With regards to an appropriate outcome, clinicians would also need to understand what "effectiveness" of a product meant, whether biofilm was reduced (if so, by how much?) or if a 100% kill was achieved. Importantly, any reductions or killing of a biofilm would need to be associated with a reduction of infective symptoms and improved patient outcome.

For a change to happen, clinicians need to start asking if the patient has a chronic biofilm or an acute infection. In orthopaedic-device, catheter or cardiac valve related infections, clinicians are fully aware of the presence of biofilm. In fact, treatment is directed at biofilm with well-documented anti-biofilm activity such as fluroquinolones [422,423] or rifampin [254], often in combination with another antibiotic [424] since resistance to rifampin can occur with a single point mutation [425]. In surgery, more aggressive debridement is also being practiced as an anti-

biofilm strategy, treating biofilm infections more like an aggressive cancer which can come back with devastating consequences unless completely eradicated. However, specific biofilm targeted treatment options are limited and if the biofilm is not eradicated there is an increased probability of generating resistance, leading to further complications for treating the infection down the road. This translates into demand for new strategies/treatments to cure biofilm infections.

Aiding in the confusion and lack of association between biofilm and chronic infection are the huge disparity in diagnostics. There are no diagnostic tools or biomarkers to help identify when biofilm is the driver of infection [93,342]. In the age of science-based medicine, how can clinicians be expected to deviate from standard measures of treating planktonic infections based on antimicrobial stewardships and make decisions to treat the infection as a biofilm infection, if there is no way to verify it?

When medical devices companies decide to pursue anti-biofilm strategies they are faced with the barriers of navigating the minefield of regulatory standards. In this instance, regulatory agencies want clinical data that demonstrates a new drug or device's ability to decrease infection rates in patients. Historically, the regulatory tests to make these claims have been based on the minimum inhibitory concentration for planktonic microorganisms (Clinical laboratory standards institute (CLSI), M02-A12, M07-A10, M100 –S26). This is different than showing that a device prevents and/or reduces biofilm. Although logically, a person cannot develop a biofilm-based infection if no biofilm forms, but this is not the outcome that is being regulated or monitored by clinicians.

Even though researchers have demonstrated that biofilm is the root cause of many chronic infections there is limited clinical biofilm data because clinicians do not routinely collect samples for biofilm specific diagnostics. Granted this would be extremely challenging, but with advancements in new non-invasive technologies, the possibility certainly exists that a mechanism for collecting these samples will exist in the future. This can be exemplified in chronic non-healing wounds complicated by biofilm, where in general practice the clinician does not collect a swab or tissue sample of the wound bed to quantify the biofilm to direct antimicrobial therapy to treat the infection.

Based upon data from industrial research, bacterial counts in the process water do not necessarily correlate to counts on the pipe's surface. This could also hold true in the human body. A low count in the urine does not mean that no biofilm is present; it just means that the biofilm has not grown to the point where the body is showing signs of infection. And of course, it would be unethical to do a study where catheters are removed over time to record the biofilm that forms and correlate this number to when the "typical" person begins to show signs of an infection (which is what occurs in industrial models for biofilm testing).

However, it is only useful to develop biofilm specific sampling if clinical microbiology has the tools for appropriate diagnostics. Currently confocal microscopy is considered the most direct way of demonstrating biofilms in clinical specimens [120] but these methods are time consuming and require highly specialized training.

This leads to a very important question. We do not know what the necessary log reduction in biofilm bacteria is that will ultimately cure the infection. For testing measures pertaining to the performance standards of an antimicrobial against planktonic microorganisms, the

necessary reduction in microorganism counts has been defined as a greater than 3 log reduction (If the reproducibility standard deviation is 1 log10 then the antimicrobial must achieve a greater than 4 log10 reduction) [426]. Without knowing what this reference value is for biofilm-based infections, a conservative approach maybe for regulatory agencies to require that the antibiotic/device must kill everything.

If this were the case what does this mean for antimicrobial therapies currently employed against biofilm that exhibit only partial reductions against biofilm *in vitro*. Using the concept of rifampin, the more commonly employed antimicrobial against biofilm, evidence has identified it does not entirely kill the bacterial biofilm in most *in vitro* models [254,427]. Does this account to regulatory agencies not validating its future use against biofilm-associated infections, and or clinicians not using it for the same purpose? Presently the simple answer is no, clinicians will still likely use this systemic antimicrobial, evidence or not, because right now that is their only option and clinicians are committed to trying to help the patient to the best of their ability.

Currently, the FDA would also not register an antibiotic or new drug based solely upon *in vitro* data as they are limited and do not consider a human immune response, nor can any statements be made about preventing or curing infection. Ideally the systemic antimicrobial is working in combination with the person's immune system to resolve the infection, but the presence of biofilm can restrict this response [428]. This may explain why people with chronic biofilm infections require multiple doses (and combinations) of systemic antimicrobials over extended periods of time (sometimes even in the order of magnitude of months or years). This exemplifies the main underlying theme of this review paper, which is that current *in vitro* biofilm tests are inadequate, and therefore are not predicting how the antibiotic will perform clinically. This may explain why there is little correlation between in *in vitro* and clinical

results. The requirement for better designed (more clinically relevant) *in vitro* biofilm tests that have gone through a standardization process may improve clinical outcomes and help direct clinicians to using antimicrobials with proven efficacy against biofilm. Once the standard methods are in place, then correlation to clinical data and ideally performance standards will follow.

7.2.5 FIVE-YEAR VIEW

Is there a clear path towards the direction of standardized approaches to biofilm strategies? Many examples outlined in this review article highlight the biofilm specific issues that need to be addressed to help provide better guidance to clinicians managing biofilm associated infections. When the performance of an anti-biofilm strategy relates to the clinical care of patients, there is a need to achieve a standardized biofilm method “utopia”. This will provide pharmaceutical / device manufacturers all the experimental parameters required so that a collaborative study may be done. From a regulatory perspective, this would also allow for the method’s reproducibility standard deviation (SD) to be determined. This requirement is highly relevant for clinicians to appreciate, who may read a paper on a new technology that performed fabulously in a one laboratory study, did fine in an animal model, but failed miserably in a clinical trial. If an appropriate statistical analysis had been performed the probability of failure would have been predicted. In general, a large percentage of experiments may lack the statistical attributes that are required of a standard method, and without statistics, there is no statistical confidence in the outcome.

In the same instance their needs to be delineation between absolute standard methods and research methods, with the latter affording the flexibility for researchers to advance new

therapeutic strategies towards biofilm-associated infections. Roberts *et al* (2015) [122] referred to this notion, suggesting that researchers should not be afraid of undertaking initial in vitro screening (non-standardized experiments). In doing so, this may provide greater predictive power for in vivo activity, and allow side-by-side comparative studies with established antimicrobial agents. This may enhance the capability to better understand biofilm associated infections.

However, Roberts et al (2015) make the same conclusion as we would, which is the most relevant system should be used based upon the questions being asked. Although preliminary experiments will allow researchers to make advances in our basic understanding of these biofilm infections, regulatory agencies require data collected with methods that have been statistically validated, which generally means the method has gone through a standardization process. Perhaps it is the reluctance of medical researchers to use standard methods that has provided a roadblock and explains why the medical field lags the biocide/industrial field with regards to biocide claims.

7.2.6. KEY ISSUES

- Biofilms show remarkable tolerance to many forms of treatments and the host immune response.

- The lack of correlation between conventional susceptibility test results and therapeutic success in chronic infections maybe reflective of biofilm presence.

- The absence of a “target” reference value required to ascertain the “effectiveness” of anti-biofilm strategies to clear infective microorganisms suggests complete eradication is required.

- A potential way forward for performance testing could be to develop a simplified biofilm assay that allows standardized adaptations (calibrated) to test parameters allowing the performance of a product to aid in predicting successful *in vivo* outcomes.

- No *in vitro* test provides a prediction on how well a product will work *in vivo*, but it does provide confidence to move forward onto animal models or costly clinical *in vivo* trials.

- Many areas of confusion regarding anti-biofilm strategies still exist for clinicians who are caught either finding it difficult to understand how *in vitro* methods translate to something of clinical relevance or think that successful *in vitro* outcomes will provide similar results *in vivo*

CHAPTER 8

FINAL DISCUSSION, LINKING OF DATA AND FUTURE WORK

8.1. 16S RDNA NEXT GENERATION SEQUENCING

Summary of key findings

When taken collectively, the genomic data presented in this thesis provides an extended view of the microbiota of infected tissues from the feet of people with diabetes. The data is comprehensive in providing an extended view of the microbiota in contrast to conventional culture methods, yet at the same time provides a conundrum in deciphering what the importance of these additional "hidden" microorganisms may be. "So, what" if a DFU has fifty bacterial genera colonising it. What does this mean? Does this affect clinical outcomes? How does this data provide alternate insights into the microbiota of DFI in comparison to conventional culture data?

In short, this genomic data (utilising 16S rDNA next generation sequencing) has identified many similarities with respect to the likely pathogens of infection when compared to culture-dependent approaches. This seems to suggest that anti-infective practices based on conventional culture data are appropriate for most patients. For example, pathogens of infection similar to those found by conventional culture were identified and illustrates the predominant role of pyogenic cocci (*Staphylococcus aureus* and *Streptococcus* spp.). This data supports

many expert guidelines that suggest anti-infective therapy should be directed towards these microorganisms.

Skin flora or pathogens of infection?

Corynebacterium spp. is often cited as commensal skin flora and ignored as a potential pathogen of infection, yet these aerobic Gram-positive bacilli were identified in most infected DFUs, contributing as one of the major taxa. Another group of microorganisms that has been poorly defined in DFI are anaerobes, because the ability to culture these microorganisms in conventional microbiology laboratories requires exhaustive measures. By circumventing conventional microbiology and utilising DNA sequencing, fastidious anaerobes (and other anaerobes) such as *Clostridiales family XI* (*Finegoldia* spp., *Anaerococcus* spp., *Peptoniphilus* spp.) were found to be prevalent in up to 80% of infected tissues and contribute as major taxa. The high relative abundance of *Corynebacterium* spp. or anaerobes in infected tissues suggests they may contribute as part of polymicrobial infections.

At a clinical level, many first line antimicrobials of choice for DFI are active against these microorganisms. As such there may not be a requirement to target them specifically unless mono-microbial cultures are identified, or infective episodes are not responding to first line therapies (in the presence of known antimicrobial susceptibilities). In the clinical data from patients enrolled in Chapter 3, no trends were observed towards an increased likelihood to fail therapy in the presence of a high abundance of anaerobes. This supports the assumption that many first line antimicrobials used for DFI are active against anaerobes.

Additionally, DNA data from patients with infected DFUs where diverse polymicrobial communities that included both pyogenic cocci and anaerobes, are examined, treatment with narrow spectrum antimicrobials resolved some of these infective episodes. Studies to determine the most efficacious anti-infective regimes for DFI are limited and there have been no publications to date to explore if broad spectrum antimicrobials improve outcomes over narrow spectrum antimicrobials. The limited sample size of 39 patients did not allow the exploration of this concept and while the previous paragraph leaves more unanswered questions than answered ones, this presents a scope for further investigation with trying to better understand why some polymicrobial infections seemingly resolve with narrow targeted therapeutics.

Most foot ulcers are chronic long-standing wounds that develop acute infections?

An important clinical scenario identified from Chapter 3 is the matter of DFU duration, and how this may help guide clinicians in determining the most appropriate ant-infective therapy. The duration of ulceration prior to infection was a major driver in determining the microbiota of infected wounds. For most DFIs, the microbiota was rich and diverse, but a pattern was identified where newer DFUs (<6 weeks) presented with fewer microorganisms that were typically a high frequency taxa profile or mono-microbial. This suggests that clinicians could safely use narrow spectrum antimicrobials directed towards aerobic Gram-positive cocci. Conversely, DFUs of longer duration (>6 weeks) had complex polymicrobial communities with high to low or low frequency taxa profiles suggesting that broad spectrum antimicrobials maybe better suited in these scenarios. Ultimately the sample size of this data restricts the ability to generalise further on these concepts, but this once again provides an opportunity for further investigation.

Correlating clinical data to microbiome analysis may identify patterns of interest to target therapy

Using simple descriptive statistics, three taxa profiles emerged from patients with DFI in this thesis. These were; High frequency taxa mostly comprised of a single microorganism (±3) (i.e. monomicrobial infection), High to low frequency taxa were comprised of between one to five (±2) dominant microorganisms followed by many low frequency taxa (i.e. polymicrobial infection) and low frequency taxa comprised on average of ≥20 (±) minor microorganisms (complex polymicrobial infection).

The ability to group microbial profiles in this manner represents an attractive analysis for researchers, most notably because it allows for pattern recognitions that may prove useful for targeted therapeutics. For example, it may prove possible that high frequency taxa in DFI as identified in this thesis being predominantly composed of *Staphylococcus* spp. or *Streptococcus* spp., only require narrow spectrum antibiotics as compared to say a low frequency taxa profile. Therefore, profiling patients based on DNA data could aid antimicrobial stewardship and reduce unnecessary used of broad agents.

Profiling patient's microbiota in this thesis is not a unique analysis to apply to wound microbiota's. The ability to cluster samples based on their microbiota profiles was undertaken by Loesche *et al* (2016) who performed Dirichlet multinominal mixture (DMM) model-based approaches. This is a rigorous statistical approach, unlike the general observations of descriptive statistics performed in this thesis. However, regardless of this fact, the microbiota profiles identified by Loesche *et al* (2016) were similar to those reported herein. Loesche *et al* reported community types labelled as CT1, CT2, CT3 and CT 4. CT1 and CT2 were highly

heterogeneous with no dominant bacteria (this is reflective of high to low and low frequency profiles in this thesis), CT3 were characterized as having high relative abundance of *Streptococcus* spp., and CT4 DFUs had high relative abundances of *S. aureus* (this is reflective of high frequency profiles in this thesis).

Other clinical data that were collected from patients enrolled as part of this thesis were the classifications of infection severity from patients presenting with DFI. These are graded according to the IDSA DFI guidelines as being mild, moderate or severe infections. One could imagine that large data sets or a registry could be compiled mapping infection severity to DNA based microbial profiles. This could allow for potential analysis of patterns of presenting microbiota's based on infection severity, in order to distinguish if certain infection severities harbour similar or dissimilar microbiota's.

In this thesis, patients with severe infections in DFUs >6 weeks presented with microbiota's that were complex, diverse and lacking any predominant or major taxa/pathogen (low frequency taxa profiles). Current guidelines for the treatment of severe DFI promote the use of intravenous broad-spectrum antimicrobials to cover both pyogenic cocci and anaerobes. The data from this thesis supports this action but presents a potential area for further exploration. Only four patients with these taxa profiles presented which makes correlating clinical and laboratory data impossible. These taxa profiles are intriguing from a microbial ecology perspective because they reflect the concept of functional equivalent pathogroups (FEP) [9], where a consortia of genotypically distinct bacteria act symbiotically to produce a pathogenic community. Understanding these complex infections that may often fail antimicrobial therapies may allow for better therapeutics.

In reflection of this area of analysis, development of translating clinically useful information on taxa profiling will only be possible where large data sets exist. Future works could look at developing a registry of sorts whereby clinicians utilsing DNA based approaches for DFI could contribute to creating large enough data sets for analysis.

8.2 MICROSCOPY TECHNIQUES

16S rDNA next generation sequencing affords the ability to depict which microorganisms are residing in that given tissue section. Conventional microbiology and development of antimicrobials have been solely based on planktonic paradigms, through methods developed in the early 1800's. For acute infections, classic microbiology has defined the causative organisms as being planktonic, rapidly multiplying cells, that damage host tissue and induce a host response, that clinicians visualise as overt clinical infection. Bacteria however, can exist in two (maybe more?) phenotypic or different growth forms, that being the planktonic form, and the second as aggregated communities of slow growing cells in a biofilm form. Biofilm infections have been defined as causing alternate symptomology, in part due to their behavioral/phenotypic changes. Of particular importance are thier noted involvement in chronic, persistent infections, in varying human health and disease pathologies [93].

Summary of key findings

Because many of the microorganisms in biofilm are not rapidly dividing or metabolizing (i.e. they show slow growth or dormant like state), the conventional culture techniques required to grow them are exhaustive, if not impossible. Methods to confirm the presence of biofilm

structures in tissue samples have therefore relied upon microscopy approaches to visualise biofilm structures in situ, or to analyse the spatial organization of microorganisms.

Chapter 4 of this thesis presents a human *in vivo* study of 65 DFUs classified as infected or chronic and non-healing. Microscopy and molecular techniques that included SEM, PNA-FISH with confocal laser scanning microscopy and 16S rDNA next generation sequencing were utilised to observe the presence of biofilms in all 65 (100%) of DFUs. Biofilms were ubiquitous and contained both mono-species and complex polymicrobial communities in the same sections of DFU tissue. This data set currently represents the largest collection of consecutive samples from DFUs within the literature. The clinical significance of our findings suggests that biofilms may have a pathogenic role across a spectrum of DFU presentations.

Do we need to change the way we treat chronic wounds?

When combined with previous data in the literature, this thesis presents a strong argument to support a paradigm shift in the management of chronic and infected wounds. Management is traditionally based on treating planktonic infections that respond rapidly to single therapies. Sessile microorganisms residing as biofilm communities are highly tolerant to these treatments and may not respond in a typical fashion to therapies that are short in duration and narrowly focused. For changes to occur in management, clinicians must begin to discuss and acknowledge the potential that biofilms contribute to infective processes in wounds. Currently this discussion does not occur and recent antimicrobial stewardship guidelines for wound-care omit any reference to biofilms [328].

For patients presenting with acute wounds that develop acute infections, the above planktonic based approach is most certainly appropriate, but the evidence presented in this thesis suggests that many patients present with chronic wounds that have acute infective episodes containing complex polymicrobial communities residing in biofilms. Using planktonic approaches for culture and establishment of sensitivities to anti-infective agents may not provide the best possible clinical outcomes. Anecdotally, as a clinician, chronic wound infections often require prolonged therapy and multi-faceted wound-care approaches. The major problem in driving changes to anti-infective practices has been the limited evidence-base for studies that have attempted to delineate treatment using either a planktonic based approach or biofilm-based approach. The area of treatment of chronic wounds presents an ideal avenue for further research both from an antibiotic perspective and from a topical antimicrobial wound-care standpoint.

Duration of the wound in a person with diabetes; does it predispose to greater opportunities of biofilm formation?

The majority of biofilms visualised *in vivo* chronic wounds in this thesis, were of longer duration (>6 weeks). However, biofilm was also identified in five DFUs of shorter duration (<6 weeks). The visualization of biofilms in chronic non-healing DFUs is expected, but formation of biofilm in shorter duration DFUs may suggest that diabetes increases the risk of biofilm formation. This could be explained by several ill-defined immunological deficits attributed too underlying hyperglycaemia [18], that contributes to a poor response of neutrophils to colonizing or invading microorganisms [340], or from impairments in microbial phagocytosis [341].

The number of shorter duration DFU samples with biofilm presence are too small to draw valid conclusions, but this presents an interesting trend that warrants further exploration.

As previously described, planktonic microorganisms have been labelled as the drivers of acute infections. In 39 infected DFUs sampled by microscopy techniques, biofilm structures were visualised. One question as a result of these findings is at what level of microbial infection do biofilms involve themselves? This data highlights a possibility whereby wound biofilm act as reservoirs for pathogens and biofilm dispersal increases the presence of planktonic microorganisms [342], that may be opportunistic or known pathogens [429]. Thus, acute infective wound symptoms may arise directly from a nidus chronic pathology.

To support this idea, the most abundant bacteria identified using 16S rDNA sequencing was *S. aureus*. PNA-FISH species-specific probes for *S. aureus* confirmed *S. aureus* as being present in the clear majority of samples as dense microbial aggregates. *S. aureus* has long been cited as the most common pathogen of infection in diabetic foot infections from culture-dependent studies [42], and here it is identified as being the predominant pathogen of infection in the presence of visualized biofilm. This data may help with regards to the development of future anti-biofilm strategies for DFU treatment where researchers should consider that anti-biofilm strategies target or be effective against *S. aureus*. This is one of the primary reasons why an ATCC strain of *S. aureus* was utilised for all the *in vitro* testing as outlined in chapters 6 & 7.

In addition to testing commonly cited microorganisms such as *S. aureus*, PNA-FISH and 16S rDNA next generation sequencing highlights the ability for wounds to also contain complex polymicrobial communities existing as biofilms. This adds to the potential complexity for anti-biofilm research that must further consider using *in vitro* or *in vivo* animal biofilm models that are clinically relevant and include polymicrobial species.

8.3 TESTING THE EFFECTIVENESS OF TOPICAL ANTIMICROBIALS USED IN WOUND PRODUCTS AGAINST BIOFILM

Within the literature, a handful of research teams have developed *in vitro* and animal biofilm models incorporating polymicrobial species [150,385,415,430]. A limitation of these approaches has been the lack of standardisation between models, with each model varying significantly. This makes comparing the results from each model impossible for clinicians, albeit the uptake of these varying models in general has been limited by both researchers/clinicians. The major reason for this has been the inherent difficulty of establishing polymicrobial models [384,385,431]. The challenge lies in culturing common (and uncommon) microbes that are found together in wounds and replicating these interactions *in vitro*. Often, co-culturing different species of microorganisms can result in the undesired killing of one or more species, even though they coexist stably in their natural environment [430].

One of the specific aims of this thesis was to develop a protocol that allowed human *in vivo* investigation of topical antimicrobials and their effects on wound biofilm, thus circumventing the requirement to perform either *in vitro* or animal model testing. The major rationale for pursuing this pathway was the large gap in the available literature, with no previous reports on human *in vivo* testing for wound related antimicrobials using advanced molecular and microscopy approaches. In chapters 6 & 7, the ability to undertake human *in vivo* trials is demonstrated. To the best of my knowledge this represents the first data in the literature which utilises a combination of molecular and microscopy approaches to better understand the effect of topical antimicrobials on human wounds.

Topical Cadexomer Iodine ointment

In chapter 6, the effectiveness of Cadexomer iodine was tested against DFUs complicated by suspected biofilm. Cadexomer iodine is a topical antimicrobial used exclusively in woundcare and was chosen for analysis based on its demonstrated superior efficacy against microbial biofilms when tested *in vitro* and in animal models, when compared against other topical antimicrobials [294,297,347]. In total 17 patients with chronic non-healing DFUs were recruited for analysis. The presence of dense biofilm was visualized and confirmed in all 17 participants using SEM, FISH or both. Following one week of application, qPCR determined the application of Cadexomer iodine resulted in 11 samples achieving up to and greater than a 1-log10 reduction.

The richness and diversity of chronic non-healing DFUs pre-and-post treatment were explored using 16S rDNA next generation sequencing. This identified that the majority of chronic DFUs enrolled into the study, harboured polymicrobial communities, with Cadexomer iodine showing broad spectrum activity. In six patients, topical antimicrobial therapy failed to reduce the microbial load. Post-treatment analysis identified diversity shifts with increasing environmental contaminants that were present in pre-treatment samples as low frequency taxa. The microorganisms increased in relative abundance post-treatment and included in Proteobacteria-E01-9C-26 Marine Group, Proteobacteria-ARKDMS-49, Archaea-Cenarchaeum, Elizabethkingia spp, Bacteroidetes-Rhodothermacae and Proteobacteria-Rhodothalssium. The microorganisms listed above are identifiable only by molecular methods (in the majority), with some of these cited as known extremophiles, existing in hostile, niche environments.

Considering the accepted notion that biofilms are tolerant to antimicrobials and combining the potential attributes of extremophile (and non-extremophile) microorganisms, this may explain why some wound microorganisms were tolerant to treatment. Post-treatment microbiota analysis identified these microorganisms increased in abundance from previously low numbers, when skin flora microorganisms such as *Pseudomonas* spp., decreased in abundance following treatment (indicating Cadexomer iodine were effective). This diversity shift may have occurred as nutrient availability increased or where mutual benefit arose.[14]

Topical Antimicrobial wound solutions

In Chapter 7, the evaluation of topical antimicrobial solutions used to clean wounds were evaluated *in vitro*, *ex vivo* porcine skin explant model, and *in vivo* human DFUs as described above. The rationale for performing this evaluation were based on clinical experiences of many woundcare/medical devices companies reporting the effectiveness of such products against biofilm, whilst the accompanying literature presented was poor to low evidence. A major problem for many medical device companies are the limited data on their wound related products. The majority of evidence is further restricted to *in vitro* testing, using non-standardised models that are not clinically relevant.

The *in vitro* biofilm model undertaken in chapter 7 tested both *P. aeruginosa* and *S. aureus* (clinically relevant wound pathogens) against varying topical antimicrobials at short exposure times of 15 minutes (clinically relevant). The results demonstrate variable efficacy in reducing biofilms, ranging between 0.5 log10 reductions to full eradication. Repeated instillation (mimicking multiple wound cleansing applications) of hypochlorous acid for 10-minute exposures in a porcine model achieved < one log10 reduction. Lastly, the application of 4 %

w/v melaleuca oil *in vivo*, resulted in no change to the total microbial load of DFUs complicated by biofilm over a seven-day period.

The results suggest that the performance of topical solutions is poor when challenged against mature biofilms using short exposure times that mimic real clinical usage (i.e. 15-minute application). The study design also identifies that antimicrobial solutions require greater exposure times to be more effective against mature biofilms. This is supported from the 24-hour exposure *in vitro* arm, whereby log10 reductions increased for most solutions.

16S rDNA next generation sequencing was performed to understand the effects of a topical antimicrobial on microbial communities *in vivo* chronic wounds. Overall, antimicrobial treatments caused a minor decrease in the diversity of wounds, shifting the residing communities to those microorganisms that were aerotolerant. This reduction in the relative abundance of anaerobe microorganisms and other low frequency taxa were not associated with reductions in microbial load. This was because for a large percentage of samples the relative abundances of both *P. aeruginosa* and *S. aureus* increased post-treatment within each wound. This suggests the diversity shift towards more dominant species such as *Staphylococcus* spp. was in response to increased nutrient availability (that resulted through removal of competing microorganisms) [14] thus sustaining the microbial load within tissues.

In contrast, Cadexomer iodine that is applied to the wound bed for a 48-72 hours exposure time, is able to achieve a >1-log10 reduction, whilst community diversity remains fairly constant. This is likely because the broad-spectrum activity of Cadexomer iodine is able to affect multiple microbial cells instead of targeting or being effective against certain microorganisms.

Is any topical antimicrobial woundcare product better than the other?

Regardless of both *in vivo* results, no topical antimicrobial tested in this thesis demonstrated a high efficacy in eradicating *in vivo* polymicrobial biofilms. This supports the requirement for further research into more effective anti-biofilm strategies. The findings also suggest there is still much to understand regarding the effects of topical antimicrobials on wound biofilm/microbial communities. Furthermore, the presence of complex polymicrobial communities that harbour uncultivable/unusual microorganisms are likely a reflection of both the longevity and continual exposure of wounds to the external environment. However, the observed community diversity shifts from common flora that typically inhabit skin to uncultivable/unusual microorganisms or low frequency taxa, suggests in some scenarios disruption to a community could bring about a negative impact on the wound.

8.4 FUTURE WORK

The breadth of this thesis has included the exploration of complex microbial communities to visualising the presence and composition of wound biofilms to testing the effectiveness of varying antimicrobial agents used in woundcare. This has produced a significant amount of data, yet at the centre of this thesis has been a primary aim to translate these findings into something of clinical relevance. In some respects, more questions outweigh answers, however undertaking this thesis has provided me with unique perspective in the areas I feel hold significant value going forward in the study to better understand diabetic foot infections. These works can be broken down into the below categories:

1. Translate microbiome evidence into practice;

Rapidly increasing antimicrobial resistance and the advent of antimicrobial stewardship is a reflection in the urgent requirement to alter how we prescribe antibiotics to patients with infections. DFIs are a classic example of patients who often receive multiple courses of anti-infectives, for prolonged periods and of broad spectrum. Most current guidelines including eTGA guidance materials propose using broad spectrum antibiotics for new infections until culture results are available to target therapy. No evidence is available to explore if outcomes for wound infections are improved based on the spectrum of activity or if new wounds of mild to moderate infection classification could be treated effectively using narrow, targeted empiric therapy.

Using16S rDNA next generation sequencing, this thesis has identified that pyogenic cocci (*S. aureus* and *Streptococcus agalactaie*), *Corynebacterium* spp., and obligate anaerobes most frequently reside in infected DFUs (albeit our understanding of the latter are the matter of debate). Some DFUs are monomicrobial, but most are polymicrobial, yet clinical correlation regarding the spectrum of activity of antibiotics, identified patient outcomes of infection resolution or failure were similar between groups. This supports the potential to undertake a clinical trial exploring the concept of narrow versus broad spectrum antibiotics for DFI and outcomes. Analysis of infected tissues using molecular and microscopy approaches would provide a robust assessment of longitudinal samples in a temporal analysis of the effects of antibiotics on microbial load, communities and presence of pathogens.

2. In diabetic foot infections, better understanding microbial infection and the host response by employing "Omics" (metagenomics, transcriptomics, proteomics);

DFI is a complex pathology to manage, with many patients requiring lower extremity amputation due to failure of therapy. Treatment failure may occur due to uncontrolled infection, signaling a poor response to antimicrobial therapy or a reflection of the poor host response to microbial invaders. To better understand DFI, metagenomic may help us to explore; the interactions of microbes in polymicrobial communities, better depict microorganisms, fungi, viruses residing in infected tissues, explore host-microbe interaction. Other research fields have applied these techniques and have differentiated known genes for protein coding sequences from which the biological functions such as pathogenicity, virulence, antibiotic susceptibility or metabolic pathways of a specific microbe-host interactions (Transcriptome and Proteome). As such this technique may help in understanding "who" did it? and aid in providing information on how we should or could direct therapy.

3. Develop / explore / research new anti-biofilm strategies;

Over the last decade many research teams/collaborations have focused their efforts towards exploring new compounds, peptides and combination therapies, that possess specific mechanisms of action against microbial aggregates or termed "anti-biofilm". One potential avenue for example, is biofilm dispersal which presents an opportunity to trigger biofilm degradation with cells reverting to planktonic susceptible forms. A review by Fleming & Rumbaugh (2017) [121] details approaches to dispersing medical biofilms, yet they summarise that some of the hurdles to development have been the lack of *in vivo* testing.

Here in presents a unique opportunity for future research and collaborations. The works undertaken in this thesis highlight the ability to perform human *in vivo* clinical trials on potential new agents. New therapies that are effective at eradicating (or removing enough biofilm to resolve infective episodes) biofilms are needed. The data presented in this thesis is testament to this. Many topical antimicrobials demonstrated in my work that are used in woundcare are traditional antiseptics based on historic formulations. They broadly target varying microbial function. Because biofilms present several mechanisms of enhanced tolerance such as the presence of EPS, the likelihood is that many topical therapies fail to work because they can't reach their intended targets (in sufficient quantities). This is demonstrated by my results where effectiveness of topical solutions and ointments produce variable and often poor results. The lack of effectiveness of many topical antimicrobials used in woundcare highlights the requirement to focus on anti-biofilm strategies. Chronic infections of the skin and soft tissue in the form of ulcers are a serious cause of morbidity and mortality, and the economic impacts to healthcare providers is significant.

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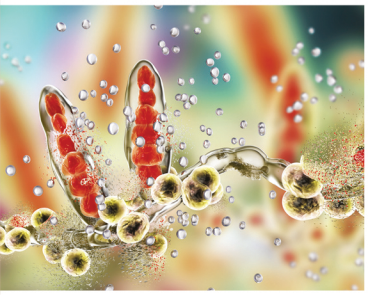
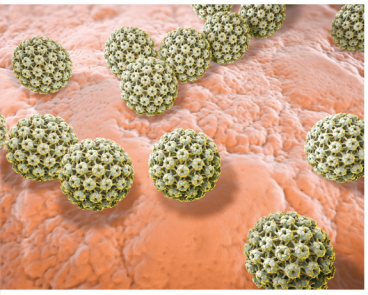
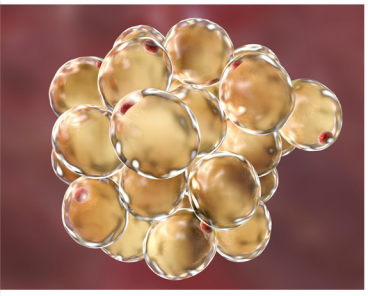
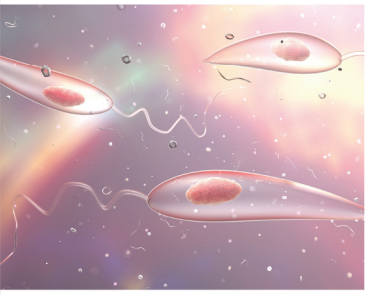
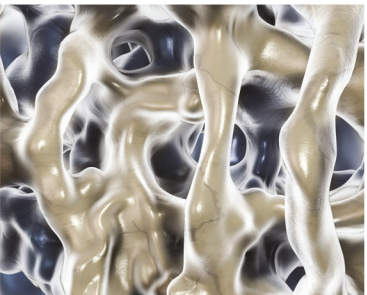
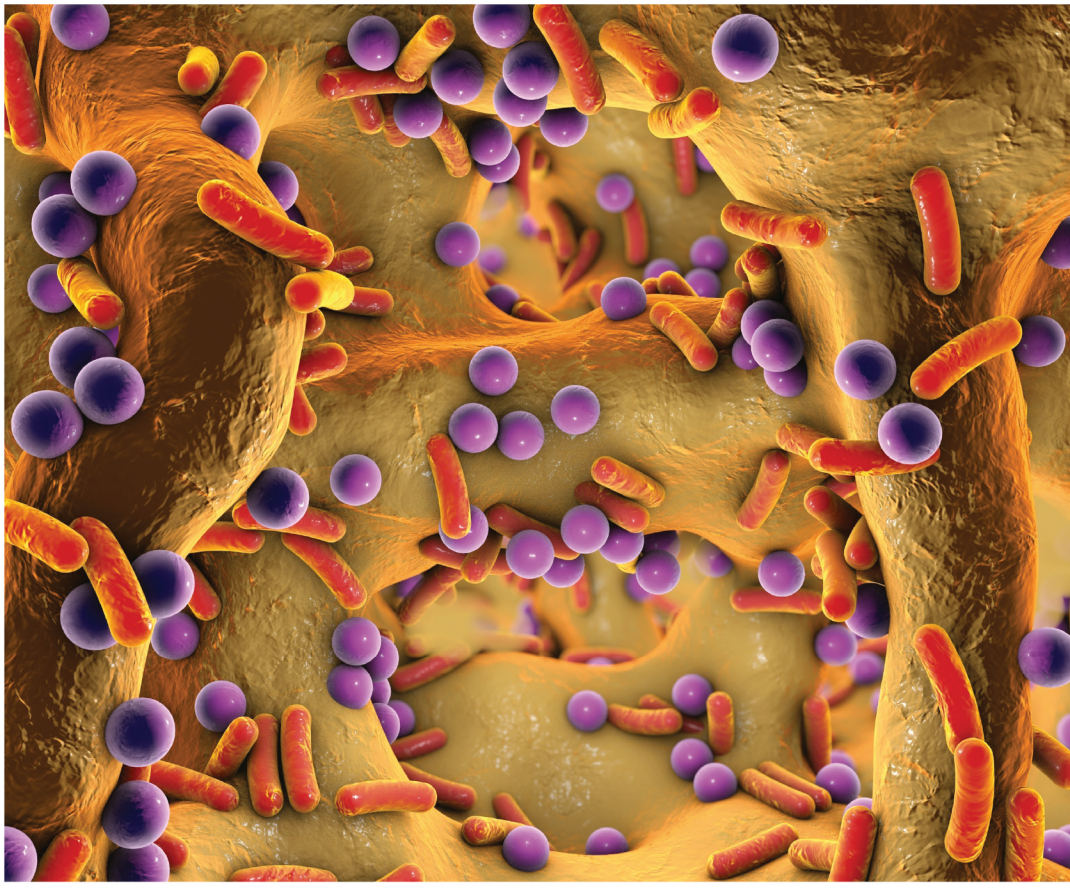






CLINICAL MICROBIOLOGY DIAGNOSIS, TREATMENT AND PROPHYLAXIS OF INFECTIONS

SERIES EDITORS: KATERYNA KON AND MAHENDRA RAI

2

THE MICROBIOLOGY OF SKIN, SOFT TISSUE, BONE AND JOINT INFECTIONS



Edited by **Kateryna Kon** and **Mahendra Rai**

CHAPTER 3

The Microbiome of Diabetic Foot Ulcers and the Role of Biofilms

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1. DIABETIC FOOT INFECTION

The most common pathway leading to a foot infection in a person with diabetes is through a physical break in the protective barrier of the skin, in the form of a diabetic foot ulcer (DFU).[1] Once the skin is breached, a DFU exposes the underlying soft tissues to potential bacterial colonization with infection arising if certain conditions favor bacterial replication. Further contiguous extension to deeper structures may produce scenarios where failure in controlling the spread of infection leads to extensive damage of host tissue and bone, and this in part may be driven by several aspects of altered immunologic function.[2] Of particular focus is the reduction in polymorphonuclear leukocyte response to bacterial infection and the role of hyperglycemia, all being extensively reviewed.[3] This scenario optimizes the pathway to lower extremity amputation in a person with diabetes.

Bacteria that contaminate and colonize wounds likely originate from the surrounding skin flora but other sources include the environment and endogenous mucous membranes, such as the gastrointestinal tract or nares.[4] One reason for this is that DFUs present an ideal environment for harboring microorganisms since they offer a warm, moist, nutritive home, especially if devitalized tissue is present in the wound bed.[4] The longer a wound remains open, the greater the chances of a more diverse and abundant bacterial colonization, with the type, depth, location, level of perfusion and the efficacy of the host immune response dictating the niche of colonizing bacteria.[4,5]

1.1 Colonization Versus Infection?

Bacterial colonization of wounds versus infection is an area that must be greatly appreciated by treating clinicians, so that the appropriate use of antimicrobials and adjunct therapies can be made effectively. While all wounds contain bacteria, colonization refers to a specific scenario where bacteria are presently multiplying but the sum of their actions are not enough to elicit an immune response.[4] Diabetic Foot Infections (DFIs) have been

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described as occurring when bacterial organisms proliferate within a wound (i.e., inframalleolar) and, in the course of doing so, cause substantial tissue damage that induces a host response accompanied by inflammation, which is clinical infection.[6] For this reason, the diagnosis of DFI has been promoted by expert groups as a "clinical diagnosis" using more than two clinical signs of infection: inflammation, erythema, local tenderness or pain, warmth and purulent discharge.[6]

In some people with diabetes, the overt clinical signs of infection are diminished or absent, and this may be due to the failure to exhibit an inflammatory response[7] with reports identifying that people with diabetes and infected chronic wounds express erythema less frequently than people without diabetes.[8] This has led to a clinical perspective that some chronic DFUs may have "secondary signs" of infection that include but are not limited to malodor, delayed wound closure and poor quality wound bed tissue.[7,9]

The fine line between colonization and infection can be clinically challenging and some clinicians have adopted more quantitative measures to differentiate potential "healthy" colonization from pathogenic infection by relying on the density of bacteria present per gram of tissue. Greater than 10^5 colony forming units (cfu) of bacteria per gram of tissue has been widely used as a key indicator of potential "bioburden" as the causative factor associated with delayed wound healing. This numerical indicator is based largely on early evidence from various wound aetiologies[10] and further incorporated by others,[11] yet controversy persists over whether a burden of $>10^5$ cfu of bacteria per gram of tissue is required to cause wound infection.

Kingsley[12] proposed a wound infection continuum model that placed an emphasis on the progression from colonization of bacteria within a wound through to infection (Fig. 1).[12] An important component of the wound continuum model is the concept of "critical colonization" which refers to the multiplication of organisms within a wound without invasion or interfering with wound healing. While the concept of critical colonization is still the center of much debate, it is often used by clinicians to explain delayed wound healing in the absence of any overt clinical signs of infection and other wound-delaying variables. This concept "if true" may be of importance for clinicians, as chronic wounds with critical colonization may benefit from local and/or topical treatments such as antimicrobial wound dressings and wound debridement, rather than systemic management with the use of antibiotics; however, no quantifiable data exists to prove or disprove this theory.

1.2 How to Obtain Adequate Cultures for Infected DFUs

The current method to identify potential pathogens of infection from DFUs is by obtaining a wound culture following the removal of devitalized tissue (either by debridement or curettage) after cleansing. The most widely available method employed by many clinicians is the use of a cotton or nylon swab of the superficial tissue using the Levine

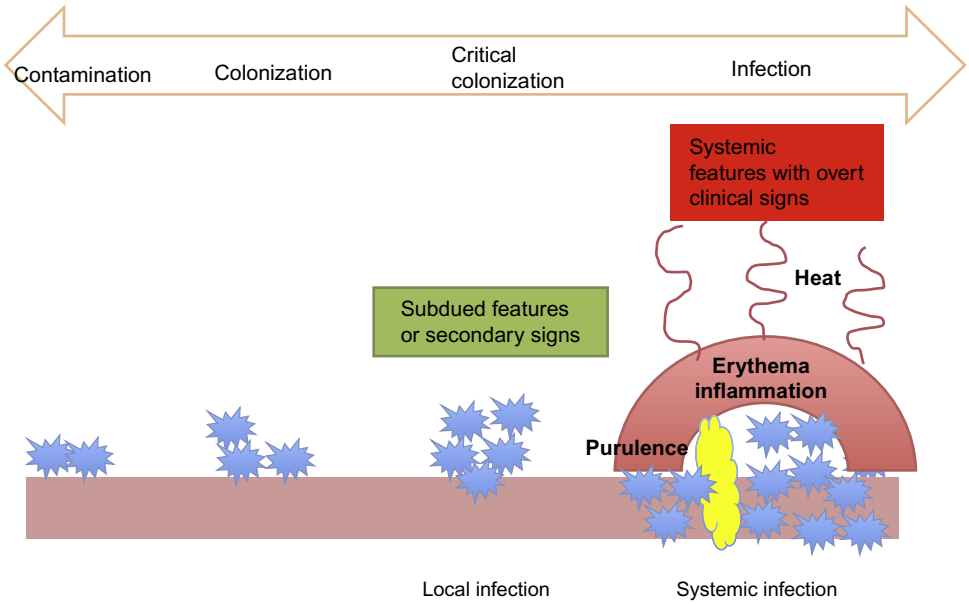

Fig. 1 A wound infection continuum model.

technique[13]; however, the concordance between superficial swabbing using the Levine technique and tissue biopsy has been reported at 78% providing only a "reasonably accurate measurement"[13] and much debate still surrounds this collection method.

The Infectious Disease Society of America (IDSA) currently propose that tissue samples are the gold standard method of collection for identifying potential bacterial pathogens from infected DFUs, as they yield the most accurate results.[6] This is exemplified by recent studies employing molecular DNA-based techniques where tissue biopsy samples from both the superficial and deep tissues of a DFU identified that the pathogens of infection were from microorganisms residing in deeper tissues.[14–16] In all instances the pathogens of infection were situated within deeper tissues and were typically fastidious aerobes along with anaerobes that were more frequently underrecognized from superficial cultures. Importantly, this supports the requirement in both a clinical scenario and for studies exploring the microbiome of wounds to utilize samples of tissue that capture both the superficial and deeper tissues through the use of a punch biopsy.

The importance in managing DFIs is underpinned by the requirement to identify any pathogen/s of infection so that antimicrobial therapy can be directed. Traditional culture-dependent methods have been utilized to identify planktonic organisms subjected to controlled laboratory conditions. The limitations of such are that culture-dependent methods select for species that flourish under the controlled conditions of the diagnostic microbiology lab and this may not necessarily reflect the most abundant or clinically important organisms in DFIs.[17]

1.3 Cultivation-Based Approaches to Defining the Bacteriology of Infected DFUs

Current culture-dependent studies on the bacteriology of DFIs have widely reported the involvement of aerobic Gram-positive cocci, mainly Staphylococcal and Streptococcal species as being the most common etiological agents that acutely infect DFUs.[18,19] In particular, the spotlight has centered on the well-known pathogen *Staphylococcus aureus* and its role in DFI has been well reported.[1,18–20]

In two large culture-dependent studies on the bacteriology of infected DFUs[18,19] samples from over 1266 patients indicated the predominant pathogens of infection as being Staphylococcal and Streptococcal species with *Enterococcus* spp. and *Corynebacterium* spp. also playing prominent roles. In both studies, anaerobes were generally reported as being of low abundance with one of the studies identifying the ratio of aerobe to anaerobe isolates being ~7:1[18] and the other study identifying the overall percentage of anaerobe isolates in only 28% of samples.[19]

Further complicating the picture of DFI, it can occur in those patients with reduced immune-mediated responses, whereby the potential involvement of low-virulence colonizers of devitalized tissue or bone such as coagulase-negative *Staphylococci* and *Corynebacterium* spp. may assume a more pathogenic role.[21] Additionally, while it is generally regarded that DFUs of short duration presenting with acute infection are monomicrobial,[1] chronic infected DFUs seem to harbor a much more complex polymicrobial flora including *Pseudomonas* spp., *Enterococci*, Enterobacteriaceae and other obligate anaerobes.[22]

1.4 Current Studies Employing Molecular Microbiology for Diabetic Foot Infections

The advent of new molecular DNA-based techniques that are culture-independent have identified limitations of traditional culture-dependent methods, acknowledging their capacity to identify a limited number of known culturable bacteria (<1%).[23]

DNA-based techniques, supported by ever-growing gene reference libraries, allow for the characterization of microbial communities or microbiomes that encompass the sum of all microorganisms residing on and within the host—bacterial, fungal, and viral.[24]

Characterizing the microbiome of infected and uninfected DFUs using molecular methods is in its infancy, and only four studies with comparable data have been published in recent years[5,25–27] (Fig. 2).

In addition to the limited work in the area of DFUs, composite evidence by authors employing molecular techniques exist for chronic wounds, where studies have pooled multiple wound etiologies to provide an overview of the chronic wound microbiome.[28–31] Most chronic wound microbiome studies to date have employed pyrosequencing centered approaches that amplify and sequence the small subunit ribosomal

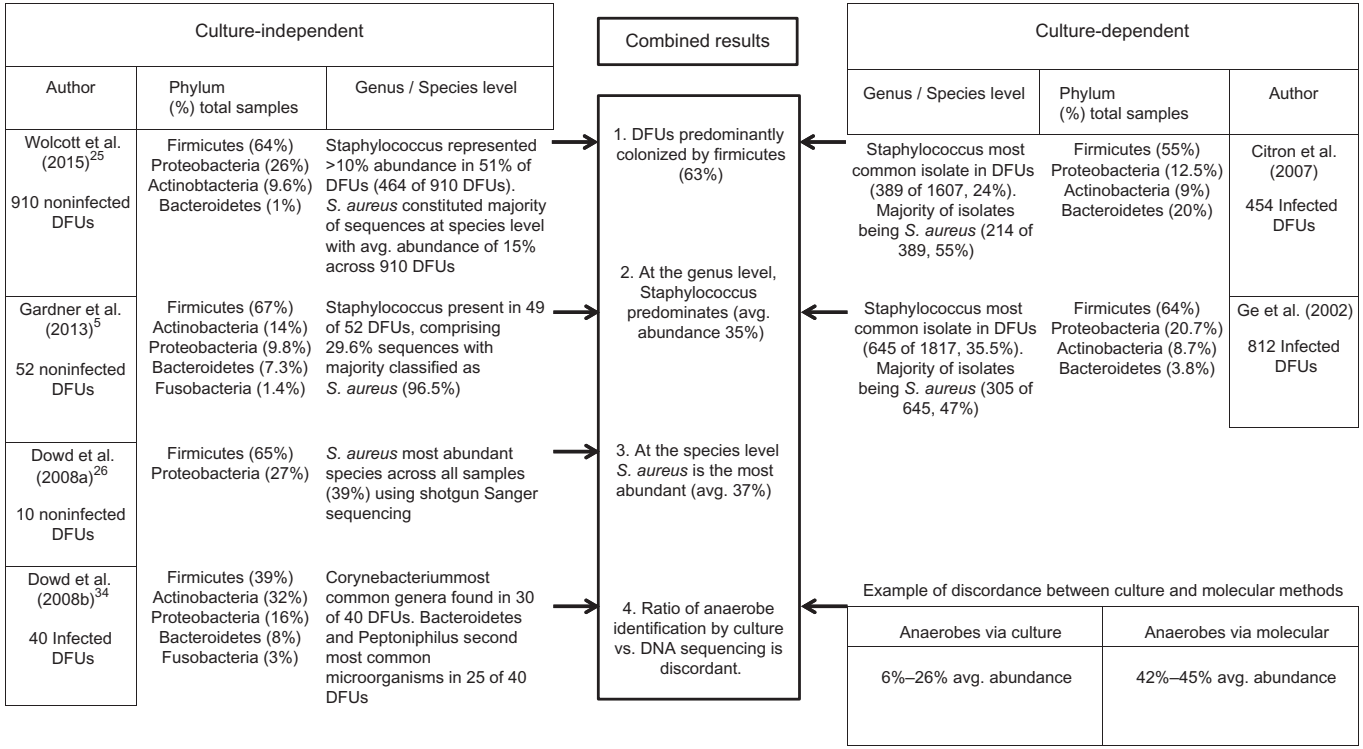

Fig. 2 Microbiome of infected and uninfected diabetic foot ulcers. *Source: used with permission from Malone M, Gosbell IB, Dickson HG, Vickery K, Espedido BA, Jensen SO. Can molecular DNA-based techniques unravel the truth about diabetic foot infections?* Diabetes Metab Res Rev *2017;* ***33****:e2834. http://dx.doi.org/10.1002/dmrr.2834. Wiley*.

RNA (16S rRNA) gene, a highly conserved gene present in all prokaryotes (bacteria) but not eukaryotes (humans). This has revealed a vastly more complex array of bacterial communities than those identified by traditional culture-dependent methods. The 16S rRNA gene is an ideal target for bacterial analysis given that it possesses nine hypervariable regions that have considerable sequence diversity between bacterial taxa.[32] The hypervariable regions are bordered by stretches of sequences that are highly conserved between bacteria, ideal for designing universal and species-specific primers to amplify the hypervariable regions by Polymerase Chain Reaction (PCR).[33]

The first report in the literature on the microbiome of DFUs was undertaken by Dowd et al.[34] reporting on 10 chronic DFUs using multiple genomic approaches that included: partial ribosomal amplification and pyrosequencing (PRAPS), full ribosomal amplification, cloning and Sanger sequencing (FRACS), density gel electrophoresis (DGGE), and Sanger sequencing (PRADS). Identification of species was undertaken using operational taxonomic units (OTUs); these are clusters of single DNA sequences grouped together based on their similarity and used to define microorganisms to a species or genus-level. DNA reads from each wound were compiled to create a pooled DNA data set from multiple DFUs. Facultative and strict anaerobic Gram-positive cocci formed the majority of sequences with genus-level identification highlighting the predominance of *Staphylococcus* spp. (PRAPS = 10,874 of 36,508 sequences, 30%; FRACS = 70 of 178 sequences, 39%) in addition to *Peptoniphilus*, *Anaerococcus*, *Rhodopseudomonas*, *Enterococcus*, *Veillonella*, *Bacterioides*, *Clostridium*, and *Finegoldia* spp., each contributing to >5% of the microbial diversity.

The second study to explore the DFU microbiome was undertaken by Dowd et al.[26] who employed bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) to sample 40 chronically infected DFUs from a range of locations on the foot and ankle. Dowd et al.[26] hypothesized that a single major pathogen would be associated with all wounds; therefore DNA reads for each DFU were reported individually and not pooled. This allowed the compilation of community profiles for each DFU, including the accurate identification of the number of samples of each genera that were detected, and the average percentage each genus contributed to its positive sample.

Results identified that each DFU contained a rich diversity of microorganisms with *Corynebacterium* (30 of 40 DFUs, avg abundance = 14.4%), *Bacteroides* (25 of 40 DFUs, avg abundance = 24.2%), *Peptoniphilus* (25 of 40 DFUs, avg abundance = 13.6%), *Fingoldia* spp. (23 of 40 DFUs, avg abundance = 6.7%) *Anaerococcus* (22 of 40 DFUs, avg abundance = 7.7%), and *Streptococcus* (21 of 40 DFUs, avg abundance = 36.5%) being present in all samples. Surprisingly, *Staphylococcus* was only present in 13 of 40 (avg abundance = 8.3%) DFUs compared to a previous study by the same authors that identified *Staphylococcus* as the major bacterial genus in 10 infected DFUs.[34] The location of ulcers originating from multiple sites of the foot and ankle may have increased the heterogeneity observed between samples in the latter study. Some DFUs may have been

purely ischemic or neuroischemic in origin, increasing the likelihood of the presence of devitalized, hypoxic tissue and favoritism towards low-virulent colonizers and anaerobic microorganisms.

No single genus of bacteria was present in all 40 DFUs and this led Dowd et al.[26] to hypothesize that the microbiome of DFUs (and chronic wounds) is dominated by multiple species that cooperate in a biofilm, a concept Dowd et al. "coin" as functionally equivalent pathogroups (FEP). This centers on genotypically distinct bacteria working cooperatively to induce pathogenesis, similar to what is observed from a known single pathogen, such as *S. aureus*; however, there is currently limited data available to support this.

Gardner and colleagues[5] profiled the microbiome of 52 individuals with noninfected DFUs using 16S rRNA gene sequence analysis and were the first to restrict the sampling of patients to a homogenous sample of DFUs (neuropathic DFUs only). The group proposed that previous studies within the area[26,28,29,34] were tainted by heterogeneous sampling (e.g., pooled samples of differing chronic wounds) and of all three DFU etiology types (neuropathic, ischemic, and neuroischemic) and, as such, these pathophysiologically distinct lesions likely harbored their own unique microenvironments and thus microbiota.

In characterizing the microbiome of 52 noninfected DFUs, a total of 13 phyla were reported from 300,660 DNA sequences with the majority being classified into Firmicutes (67%), Actinobacteria (14%), Proteobacteria (9.8%), Bacteroidetes (7.3%), and Fusobacteria (1.4%). *Staphylococcus* was identified as the most common and abundant genus in 49 of 52 DFU samples (DNA sequences = 88,995 of 300,660, abundance = 29.6%). At a species-level the majority of sequences belonged to the common pathogen *S. aureus* (*S. aureus* sequences = 85,880 of 88,995, 96.5%), an unsurprising finding considering the highly documented role of this microorganism in diabetes-related foot infection.[6]

Further analysis of microbial diversity in 52 DFUs reported on average 30 OTUs per DFU (range 7–64) in comparison to culture-dependent analysis that detected on average 4 OTUs per DFU ($P < .0001$). Comparisons of the relative bacterial abundances within each DFU using culture-dependent analysis identified the overestimation in the abundance of *Staphylococcus* spp. (0.47 vs 0.32, $P = .0001$) and underestimation of anaerobes (0.11 vs 0.18, $P = .0063$) in comparison to 16S rRNA gene sequence analysis. By culture, anaerobes were identified as the predominant organisms in only 6 of 52 DFUs (12%), a finding consistent with the known limitations of culture-dependent methods, particularly in the identification of slow-growing, fastidious anaerobic organisms.[5] In contrast, 16S rRNA gene sequence analysis identified twice this amount (12 DFUs, 23%); this is of importance at a clinical level where the role of anaerobes in DFI and wounds is often underestimated. Their significance as highly virulent pathogens, detrimental to the wound environment or as pathogens of DFI, is only becoming appreciated through advances in new molecular techniques that are culture-independent.

Gardner et al.[5] also depicted bacterial community structure using a statistical approach of Euclidean distances to determine how similar DFU attributes were to the indicators of wound bioburden (microbial load, diversity, and presence of pathogens). The partitioning of data identified three clusters, which Gardener et al.[5] referred to as EUC1, EUC2, and EUC3. EUC1 contained significantly greater OTU richness ($P=.006$), diversity ($P=.02$), and bacterial load ($P=.02$), whereas EUC2 contained a greater abundance of *Staphylococcus* and lower abundance of anaerobes ($P=.0003$), and EUC3 contained the highest abundance of *Streptococcus* ($P=.0002$).

Euclidean clusters were also mapped against clinical markers that included HbA1c, mean tissue oxygenation, ulcer duration, ulcer depth, ulcer surface area, and necrotic tissue. EUC1 containing greater OTU richness, diversity, and bacterial load, were associated with DFUs of longer duration ($P=.02$) and increased ulcer depth ($P=.01$). The EUC1 cluster also contained a greater abundance of anaerobes ($P=.01$) and Proteobacteria ($P=.005$), a likely association with wound longevity and deeper tissue involvement. EUC2 and EUC3 had the highest proportions of both *Staphylococci* and *Streptococci* with laboratory markers from these participants recording the highest levels of glycosylated hemoglobin (HbA1c %) (Median EUC 2 HbA1c = 9.2% versus median EUC 3 HbA1c = 9.4%).

Assumptions on infections in people with diabetes have repeatedly associated *Staphylococci* and *Streptococci* as major pathogens, noting higher colonization rates in those with suboptimal glycemic control.[35] A plethora of studies have also identified that hyperglycemia causes immunosuppression, and a reduction in glucose by a variety of means reverses immune function deficits.[36–38] However, no evidence exists identifying direct links between hyperglycemia, infection and potential deficits in the immune response to specific microorganisms.[6,39]

Gardner et al.[5] invested a significant proportion of their analysis on undertaking a statistical approach to partitioning DFUs into meaningful clusters based on their associations to the three dimensions of bioburden (i.e., microbial load, microbial diversity, and presence of pathogens). Interpretation and validation of cluster analysis occurred through a silhouette score (SI), a method used to report the graphical representation of how well each object lies within its cluster in comparison to other clusters. An average SI score of 0.42 was reported by Gardner et al.,[5] but an SI score of <0.5 signifies only modest support for the proposed cluster algorithms.[40,41] Wu et al.[42], who explored gut microbial enterotypes using molecular approaches, recommended using a high SI threshold (≥ 0.75) for validating clusters.

The statistical significance of partitioning 52 DFUs into similar clusters given the moderate SI score should be aired on the side of caution, as a low SI score suggests that clustering could be due to chance. Further studies aiming to increase cluster validity through higher SI scores may be required in supporting the partitioning of the DFU microbiome to clinical indicators similar to those observed by Gardner et al.

Wolcott et al.[25] retrospectively reported 910 noninfected DFUs using 16S rRNA pyrosequencing and currently represents the largest body of evidence surveying the microbiome of DFUs. Wound samples were collected from superficial debridement material obtained from the wound bed and relative abundances were calculated from species-level OTUs constituting >10% of all DNA reads per sample. Given the large sample size from multiple wound etiologies, individual wound microbiomes were not reported and DNA sequences were pooled. Of 910 DFUs, the most abundant phylum was the Firmicutes (64%) followed by Proteobacteria (26%), Actinobacteria (9.6%), and Bacteroidetes (1%). *Staphylococcus* (31%) was the most abundant OTU with *S. aureus* (48%) and *S. epidermidis* (35%) predominating at the species-level.

In review of the studies employing amplification and sequence analysis of the 16S rRNA gene to characterize the microorganisms involved in DFI, few have sampled participants with overt clinical signs of infection. It is generally regarded that clinically uninfected wounds should not be treated with antimicrobials and therefore should not be cultured.[6,43] The clinical relevance of culturing uninfected DFUs to characterize the microbiome is thus debatable, and worth expanding upon further. In patients with diabetes presenting with overt clinical signs of infection, the decision to obtain a culture from a wound to identify a pathogen of infection is clinically justified. It has been well addressed in the literature; however, many patients with diabetes do not display overt clinical signs of infection in the presence of a nonhealing DFU.[7,44]

Some clinical researchers have proposed under these circumstances to utilize secondary signs and symptoms (i.e., malodor, increased exudate, and poor quality tissue) to diagnose infections that may be more pertinent to guiding clinicians facing wounds that are nonhealing in the presence of standard care.[45] Therefore, obtaining cultures to examine the microbiome of patients with "masked" signs of infection in DFUs, that are nonhealing despite standard care, may prove greatly beneficial. With the exception of Gardner et al.,[5] who restricted sampling to a homogenous group of DFUs (neuropathic), all other microbiome studies have included the sampling of different DFU etiologies. The extent to which community diversity is affected through variances in wound etiologies is currently the focus of debate. It is generally accepted that DFUs of ischemic origin may contain a wound bed environment of devitalized, slough tissue. This may perpetuate very different bacteria from those of the healthy vascularized tissue common in neuropathic DFUs.[1,46] Further studies comparing each DFU etiology individually may clarify this matter further.

Lastly, it has been cited in the literature that certain aerobic species are often overestimated as the primary pathogens in DFI. When comparing the evidence on the bacteriology of DFIs through both culture-dependent and -independent approaches, both corroborate *S. aureus* as being the predominant pathogen colonizing DFUs. In tandem with this, reports have also suggested that the abundance and role of anaerobes are greatly underestimated. The significance, however, at a clinical level of identifying additional

"hidden" anaerobes that often form part of a polymicrobial infection is yet undefined. Many antimicrobial agents commonly used in the treatment of DFIs have a broad spectrum of activity to cover most anaerobes. Furthermore, no published studies are available reporting improved clinical outcomes when adding additional antianaerobic directed therapy to concurrent regimes.

2. BIOFILMS

During most of the history and development of microbiology, the general understanding of the role microbes play in human health and disease has been that they exist as planktonic or free-floating single-cell organisms. Seminal works by Louis Pasteur and Robert Koch in the mid-to-late 1800s paved the way in the field of microbiology, and laboratories still use the 150-year-old methods developed by these pioneers. These techniques postulate that microbial cells act in a planktonic state: that is, they disperse in a liquid environment.

However, emerging evidence from the previous century, based on microbial studies of aquatic environments and dental plaque, provided insights that microorganisms have a natural tendency to associate surfaces, preferring a sessile lifestyle.[47,48] This early work, which focused predominantly on environmental samples, later provided a platform for the contemporary medical models that we have come to understand as "microbial biofilms." Biofilms are frequently defined based on in vitro observations through methods such as scanning electron microscopy (SEM) or peptide nucleic acid-flourescent in situ hybridization (FISH) (Fig. 3A and B).

The classic definitions often describe biofilms as bacteria attached to surfaces, encapsulated in a self-produced matrix, and tolerant to antimicrobials.[49,50] The biofilm concept and its involvement in human health and disease were first described in the medical literature in 1978,[48] with much debate following. This was followed by an explosion of biofilm analysis in human health and disease in the 1980s, which was driven largely

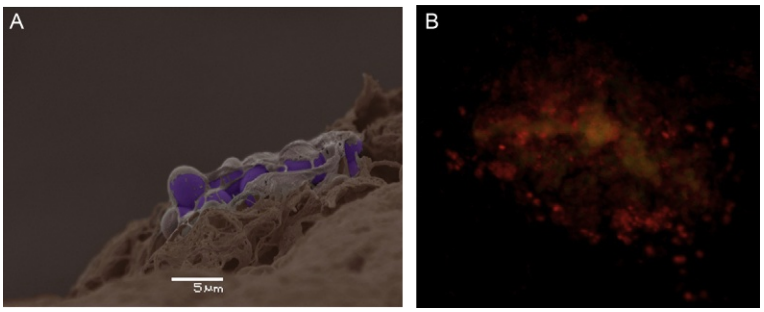

Fig. 3 Scanning electron microscopy (SEM) and peptide nucleic acid fluorescent in situ hybridization (FISH) methods for detection of biofilms (A and B, respectively).

by advancements in emerging technologies and techniques applicable to the study of bacterial populations in situ. Using light and electron microscopy, in combination with specific probes to define cell surface structures, William Costerton (1934–2012), a microbial ecologist, was credited with advancing the understanding of the role biofilms play in human health and disease.

Costerton's early enterprising works were fixed on environmental models, but this quickly transcended to the medical arena with thoughts of "how does a bacterium know whether it is in a urinary catheter or an alpine stream?" hypothesizing that the bacterium would grow as a biofilm on both surfaces regardless.[51] Costerton and colleagues were also the first to propose the role of *Pseudomonas aeruginosa* biofilms in the sputum of cystic fibrosis patients,[52] much to the energetic refutation of medical peers at the time. The concept of biofilms in human health and disease is now universally accepted in tuberculosis,[53] periodontal disease and dental caries,[54] cystic fibrosis,[52,55,56] in-dwelling medical device infections,[57] otitis media and other upper respiratory infections,[58,59] and chronic wounds.[60,61]

Early work by Costerton and colleagues[62] identified that bacteria growing on medical devices existed within a biofilm, and that they exhibited a remarkable tolerance to both host defenses and antimicrobial therapy. A plethora of in vitro biofilm models have elucidated that bacterial biofilms can withstand antimicrobial concentrations 100–1000 times higher than those of planktonic counterparts.[63–65] This would suggest that biofilm cells harbor the capability to switch on multidrug resistance mechanisms that they only express during biofilm growth. If this were true, then mutants expressing the same resistance would have been seen in rapidly multiplying planktonic cultures. This has not been the case, which has therefore led to the riddle of how a biofilm can be resistant to killing by many antimicrobials in the absence of specific resistance mechanisms.[66]

In spite of the wealth of research undertaken to identify biofilm resistance and/or tolerance to antimicrobials, no single causative mechanism has been identified. Instead, it has been suggested that a likely combination of factors contributes to biofilm recalcitrance, with several areas of interest including but not limited to: slow or incomplete permeation of antimicrobials through extracellular polymeric substance (EPS)[63] altered microenvironment and niches within biofilms promoting slow growth rates and adaptive stress response,[67,68] efflux pumps,[69] and the role of "persisters."[70]

2.1 Clinical Significance of Bacterial Biofilms in Chronic Wounds

2.1.1 How Do Biofilms Impede Wound Healing?

The exact mechanisms of biofilm impairment on the healing processes of wounds remain ambiguous. Existing data suggest a chronic wound is kept in either a vicious inflammatory state, or subject to localized low oxygen tensions. The pathways behind this are not clear, but several systemic and local factors may contribute to the occurrence and maintenance of the wound chronicity.

2.1.2 Biofilms Sustain Hyperinflammation

In a review article, Wolcott and colleagues[71] present a detailed hypothesis summarizing that once a biofilm community becomes established, its stubbornness and frequent failure to many treatments propagates hyperinflammation. Specifically, they propose that biofilm phenotype bacteria produce proteases that inhibit and destroy extracellular matrix. In addition, the chronic wound environment also possesses host-derived proteases. Together this may overfill a chronic wound with a proteolytic mix of proteases, elastases, and gelatinases (these are commonly referred to as matrix metalloproteinases, or MMPs). In parallel, biofilm adherence to the wound bed may also potentially inhibit the release of the natural suppressor of MMPs—tissue inhibitors of MMPs (TIMPS). This scenario may therefore sustain a perpetual state of hyperinflammation.

Walcott and colleagues also put forward the potential of biofilms to "bait" the immune system through releasing planktonic bacteria. Walcott and colleagues suggest that the presence of anaerobic bacteria play a key role throughout, releasing a cell wall constituent lipopolysaccharide, a potent inflammatory inducer. In an animal model on cystic fibrosis patients, biofilms forming *P. aeruginosa* were shown to undergo lipopolysaccharide modifications that induced greater inflammatory responses in mice.[72] No human in vivo data exist to support this theory for chronic wounds.

2.1.3 Biofilms May Contribute to Localized Areas of Low Oxygen Tension Within a Wound

Early microelectrode studies of aerobic in vitro biofilm models found discrete areas within biofilm that had significant oxygen depletion.[67] This suggested that areas of biofilm housed microniches favoring differing microorganisms, and may explain how the presence of anaerobes in mixed-species biofilms exist, contribute and cooperate with aerobic neighbors.

Further studies employing microelectrodes with Confocal Laser Scanning Microscopy (CLSM) have identified microdomains with different biochemical environments including alterations in pH and oxygen.[73] Recent data by James and colleagues[68] have provided further evidence to support a concept of localized low oxygen tensions contributing to wound chronicity. Using oxygen microsensors and transcriptomics (examining microbial metabolic activities) to study in situ biofilms, steep oxygen gradients and induced oxygen-limitation stress responses were identified from bacteria. Additionally, through transcriptomics, it was established that metabolic activities of the biofilm and the recruitment of cells that consume oxygen for host defensive processes were the primary pathways of oxygen depletion. Taken collectively, this data supports the concept of a biofilm establishing and maintaining localized low oxygen tensions in a wound, thus contributing to chronicity.

3. SUMMARY

The progression of infection in a person with diabetes can lead to the devastating requirement for a lower extremity amputation. Therefore the clinical identification of infection and the grading of its severity is key. To direct targeted therapy, clinicians need to understand the microorganisms involved in the process, but it is clear that molecular DNA-based approaches identify many "hidden" microorganisms. Both the progress and application of current DNA sequencing continues to rapidly evolve, but thus far we have only been enlightened with a broader view of "which microorganisms are there." The interpretation and any clinical implications of additional bacteria within samples remain unclear, but as DNA-based evidence increases we may be enlightened. Importantly, the concept of biofilms and their involvement as contributors to chronic nonhealing wounds and in the pathogenesis of chronic infection poses a significant shift in therapeutic paradigms. This needs to be approached cautiously, with input from antimicrobial stewardships, wound-care clinicians and the broader medical community.

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REVIEW ARTICLE

Can molecular DNA-based techniques unravel the truth about diabetic foot infections?

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Abstract

Diabetes foot infections (DFIs) are a common condition and a major causal pathway to lower extremity amputation. Identification of causative pathogens is vital in directing antimicrobial therapy. Historically, clinicians have relied upon culture-dependent techniques that are now acknowledged as both being selective for microorganisms that thrive under the physiological and nutritional constraints of the microbiology laboratory and that grossly underestimate the microbial diversity of a sample. The amplification and sequence analysis of the 16S rRNA gene has revealed a diversity of microorganisms in DFIs, extending the view of the diabetic foot microbiome. The interpretation of these findings and their relevance to clinical care remains largely unexplored. The advent of molecular methods that are culture-independent and employ massively parallel DNA sequencing technology represents a potential 'game changer'. Metagenomics and its shotgun approach to surveying all DNA within a sample (whole genome sequencing) affords the possibility to characterize not only the microbial diversity within a DFI (i.e. 'which microorganisms are present') but the biological functions of the community such as virulence and pathogenicity (i.e. 'what are the microorganisms capable of doing'), moving the focus from single species as pathogens to groups of species. This review will examine the new molecular techniques for exploration of the microbiome of infected and uninfected diabetic foot ulcers, exploring the potential of these new technologies and postulating how they could translate to improved clinical care.

Keywords microbiome; diabetic foot infection; DNA sequencing; metagenomics

Introduction

One of the causal pathways to lower extremity amputation in a person with diabetes is the development of a diabetic foot ulcer (DFU) that becomes infected (diabetes foot infection or DFI) [1]. In a person with diabetes, the development of a DFU is most commonly associated with loss of protective sensation (peripheral neuropathy), altered foot architecture and some form of trauma. These factors allow a physical break in the protective barrier of the skin to go unnoticed and provide an ideal environment for colonization with various opportunistic microorganisms [2]. Factors including a retarded host immune response and pathogen-related dynamics (such as virulence or pathogenicity) may predispose the DFU to further microbial replication and

invasion, resulting in damage to host tissues and an inflammatory response that is characterized as a clinical infection [3].

Expert consensus opinion has promoted the diagnosis of DFI as a clinical diagnosis using greater than two clinical signs that include inflammation, erythema, local tenderness or pain, warmth and purulent discharge [3]. Further spread of bacteria may lead to both the involvement of deeper structures with extensive damage to both tissue and bone and the presence of a systemic inflammatory response syndrome requiring hospitalization. Uncontrolled DFI may lead to lower extremity amputation in a person with diabetes.

DFU bacteriology via culture dependent techniques

Ideal management of DFIs requires identification of any pathogen so that antimicrobial therapy can be directed against them. Traditional culture-dependent methods have been utilized to identify planktonic organisms. The limitations of this are that culture-dependent methods select for species that flourish under the controlled conditions of the diagnostic microbiology laboratory, and this may not necessarily reflect the most abundant or clinically important organisms in DFIs [4].

Current culture-dependent studies on the bacteriology of DFIs have widely reported the involvement of aerobic Gram-positive cocci, with staphylococcal and streptococcal species being the most common aetiological microorganisms that acutely infect DFUs (Table 1) [5,6]. In particular, the spotlight has centred on the well-known pathogen *Staphylococcus aureus*, and its role in DFI has been well reported [2,5–8].

Two large culture-dependent studies on the bacteriology of infected DFUs sampled over 1266 patients. The predominant pathogens as staphylococcal and streptococcal species with enterococcal species and corynebacterial species also playing prominent roles [5,6]. In both studies, anaerobes were generally reported as being of low abundance, with one of the studies identifying the ratio of aerobe to anaerobe isolates of approximately 7 : 1 [5] and the other study identifying anaerobe isolates in 28% of samples [6].

Complicating the picture of DFI are those patients with reduced immune-mediated responses, where low virulence colonizers of devitalized tissue or bone, such as coagulase-negative staphylococci, and corynebacteria may assume a more pathogenic role [9]. Additionally, while DFUs of short duration presenting with acute infection are generally monomicrobial [2], chronic infected DFUs appear to harbour a much more complex polymicrobial flora including *Pseudomonadaceae*, *Enterococci*, *Enterobacteriaceae* and other obligate anaerobes [6,10,11].

Current studies employing molecular microbiology to explore DFUs

The advent of new molecular DNA based techniques that are culture-independent has identified limitations of traditional culture-dependent methods, acknowledging their capacity to identify a limited number of known cultivable bacteria (less than 1%) [4].

DNA-based techniques, supported by ever-growing gene reference libraries, allow for the characterization of microbial communities or microbiomes that encompass the sum of all microorganisms residing on and within the host – bacterial, fungal and viral [12].

Characterizing the microbiome of DFIs using molecular methods is in its infancy. Only four studies with comparable data have been published in recent years (Figure 1) [13–15]. In all of these studies, both infected and uninfected tissue or swabs were obtained from DFUs. Composite evidence by authors employing molecular techniques exists for other chronic wounds to provide an overview of the chronic wound microbiome [16–19]. Most chronic wound microbiome studies to date have employed pyrosequencing centred approaches that amplify and sequence the small subunit ribosomal RNA (16S rRNA) gene, a highly conserved gene present in all prokaryotes (bacteria) but not eukaryotes (humans). This has revealed a vastly more complex array of bacterial communities than those identified by traditional culture-dependent methods. The 16S rRNA gene is an ideal target for bacterial analysis given that it possesses nine hypervariable regions that have considerable sequence diversity between bacterial taxa [20]. The hypervariable regions are bordered by stretches of sequences that are highly conserved between bacteria, ideal for designing universal and species-specific primers to amplify the hypervariable regions by polymerase chain reaction (PCR) [21]. The workflows required to generate data on microbiome studies that include chronic wounds are depicted in Figure 1.

The first report in the literature on the microbiome of DFUs was by Dowd *et al.* reporting on ten chronic DFUs using multiple genomic approaches that included partial ribosomal amplification and pyrosequencing (PRAPS), full ribosomal amplification, cloning and Sanger sequencing (FRACS), density gel electro-phoresis (DGGE) and Sanger sequencing (PRADS) [22]. Identification of species was undertaken using operational taxonomic units (OTUs). These are clusters of single DNA sequences grouped together based on their similarity and used to define microorganisms to a species or genus level. Sequences identified both facultative

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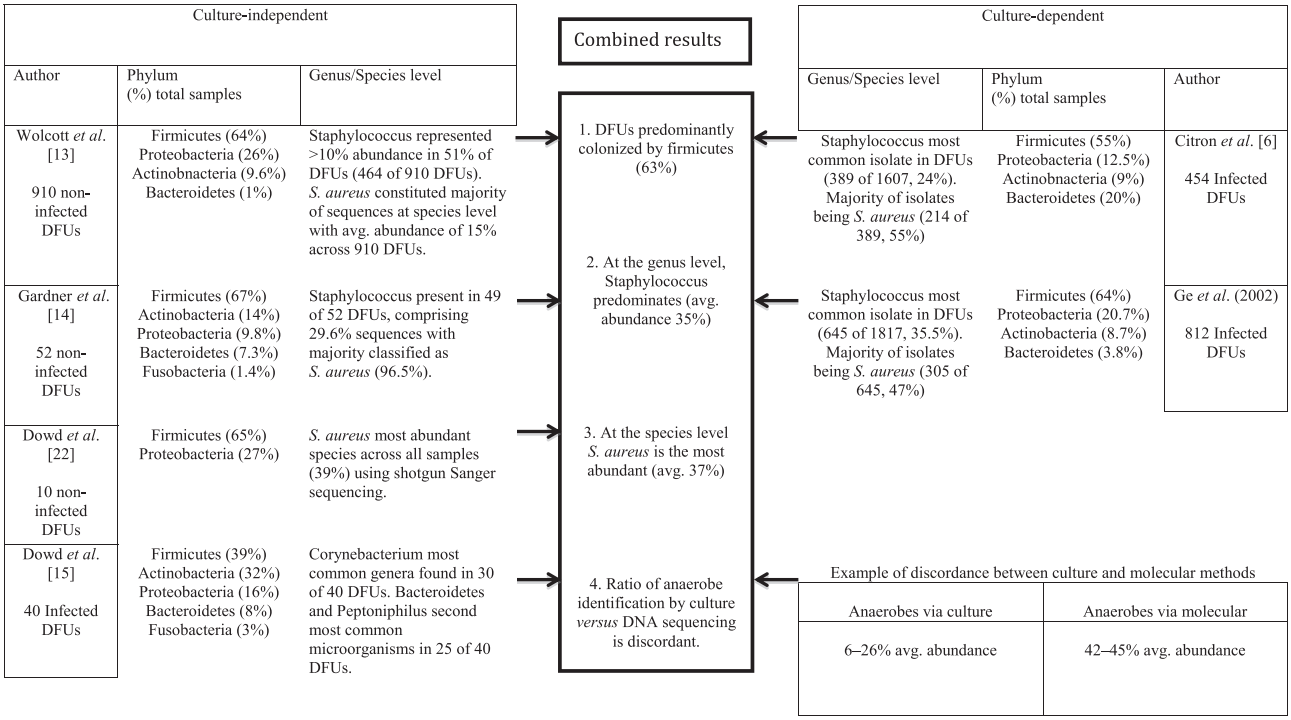
Table 1. Bacteriology of DFI. A comparison of culture dependent and independent analysis



and strict anaerobic Gram-positive cocci that included Staphylococcus, clostridales family XI (Peptoniphilus, Anaerococcus and Finegoldia) Rhodopseudomonas, Enterococcus, Veillonella and Bacterioides.

The second study to explore DFU microbiome was undertaken by Dowd *et al*. who employed bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) to sample 40 chronic DFUs from a range of locations on the foot and ankle [15]. Results identified each DFU contained a rich diversity of microorganisms with *Corynebacterium* (30 of 40 DFUs, avg abundance = 14.4%), *Bacteroides* (25 of 40 DFUs, avg abundance = 24.2%), *Peptoniphilus* (25 of 40 DFUs, avg abundance = 13.6%), *Fingoldia* spp. (23 of 40 DFUs, avg abundance = 6.7%), *Anaerococcus* (22 of 40 DFUs, avg abundance = 7.7%) and *Streptococcus* (21 of 40, avg abundance = 36.5%) being present in all samples.

Gardner and colleagues profiled the microbiome of 52 individuals with non-infected DFUs using 16S rRNA gene sequence analysis and were the first to restrict the sampling of patients to a homogenous sample of DFUs (neuropathic DFUs only) [14]. The group proposed that previous studies within the area [15–17,22] were tainted by heterogeneous sampling [e.g. pooled samples of differing chronic wounds and of all three DFU aetiology types (neuropathic, ischemic and neuroischemic)], and as such, these pathophysiologically distinct lesions likely harboured their own unique microenvironments and thus microbiota.

In characterizing the microbiome of 52 non-infected DFUs, *Staphylococcus* was identified as the most common and abundant genus in 49 of 52 DFU samples. At a species-level, the majority of sequences belonged to the common pathogen *S. aureus*, an unsurprising finding considering the highly documented role of this microorganism in diabetes related foot infection [3]. Comparisons of the relative bacterial abundances within each DFU using culture-dependent analysis identified the overestimation in the abundance of *Staphylococcus* spp. (0.47 *vs*. 0.32, $p = 0.0001$) and underestimation of anaerobes (0.11 *vs*. 0.18, $p = 0.0063$) in comparison with 16S rRNA gene sequence analysis.

By culture, anaerobes were identified as the predominant organisms in only 6 of 52 DFUs (12%) a finding consistent with the known limitations of culture-dependent methods, particularly in the identification of slow-growing, fastidious anaerobic organisms [14]. In contrast, 16S rRNA gene sequence analysis identified twice this amount (12 DFUs, 23%); this is of importance at a clinical level where the role of anaerobes in DFI and wounds is often underestimated. Their significance as highly virulent pathogens, detrimental to the wound environment [23] or as pathogens of DFI [10] is only becoming appreciated through advances in new molecular techniques that are culture-independent.

Gardner *et al*. also depicted bacterial community structure using a statistical approach of Euclidean distances to

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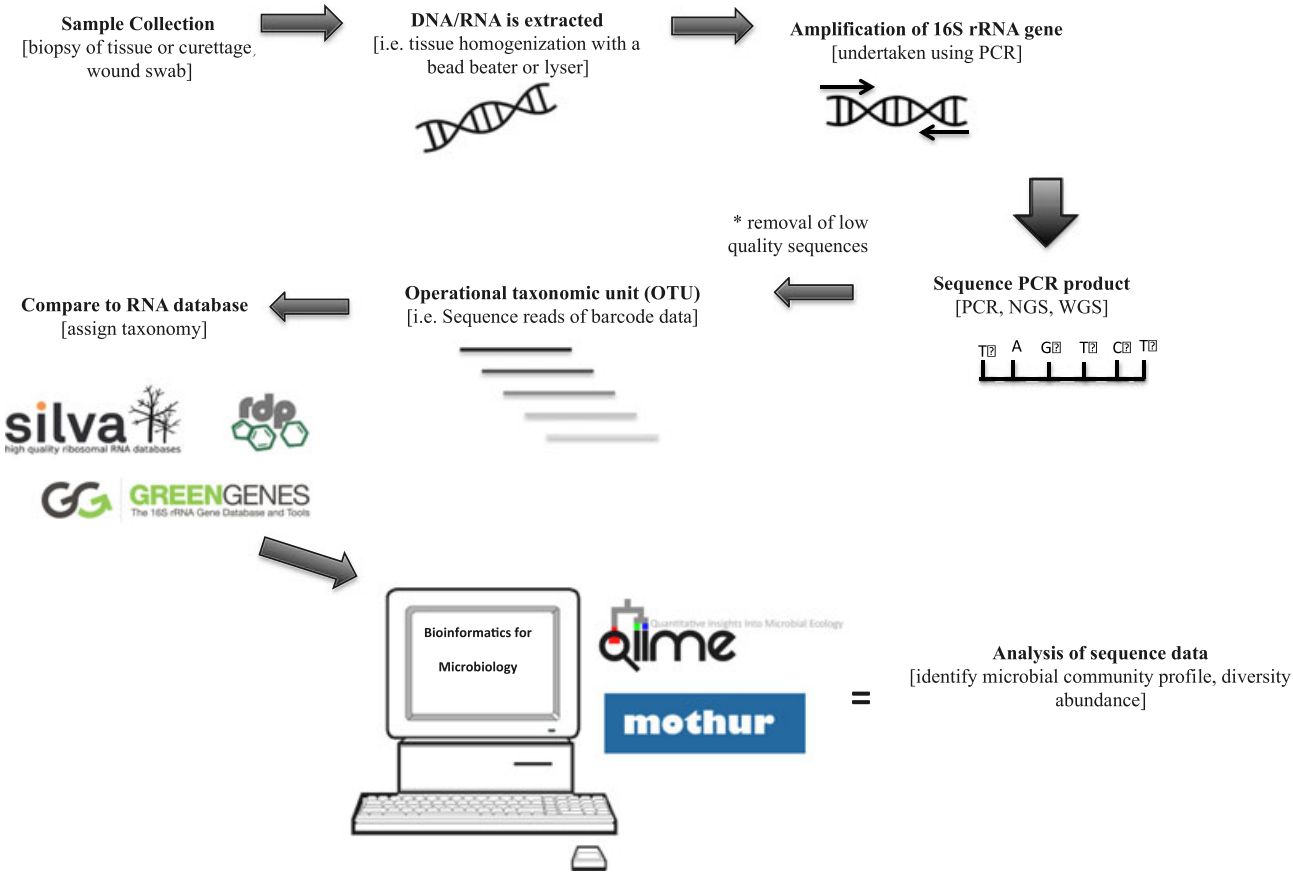

Figure 1. An overview of a common polymerase chain reaction (PCR) and massively parallel sequencing (MPS)-based workflow employed for microbiome research into chronic wounds, diabetic foot ulcers and diabetes foot infections. WGS, whole genome sequencing

determine how similar DFU attributes were to the indicators of wound bioburden (microbial load, diversity and presence of pathogens). The partitioning of data identified three clusters; EUC1 contained significantly greater OTU richness ($p = 0.006$), diversity ($p = 0.02$) and bacterial load ($p = 0.02$). This cluster was associated with DFUs of longer duration ($p = 0.02$), increased ulcer depth ($p = 0.01$), contained a greater abundance of anaerobes ($p = 0.01$) and proteobacteria ($p = 0.005$). This is likely associated with wound longevity and deeper tissue involvement.

EUC2 contained a greater abundance of *Staphylococcus* and lower abundance of anaerobes ($p = 0.0003$), and EUC3 contained the highest abundance of *Streptococcus* ($p = 0.0002$). EUC2 and EUC3 had the highest proportions of both staphylococci and streptococci with laboratory markers from these participants recording the highest levels of glycosylated haemoglobin (HbA_{1c} %) (Median EUC2 $HbA_{1c} = 9.2\%$ *versus* median EUC3 $HbA_{1c} = 9.4\%$).

Lastly, Wolcott *et al*. retrospectively characterized 910 non-infected DFUs using 16S rRNA pyrosequencing, and this currently represents the largest survey of the microbiome of DFUs [13]. Wound samples were collected from superficial debridement material obtained from the wound bed, and relative abundances were calculated from species-level OTUs constituting >10% of all DNA reads per sample. Given the large sample size from multiple wound aetiologies, individual wound microbiomes were not reported, and DNA sequences were pooled. Of 910 DFUs, the most abundant phylum was the Firmicutes (64%) followed by Proteobacteria (26%), Actinobacteria (9.6%) and Bacteroidetes (1%). *Staphylococcus* (31%) was the most abundant OTU with *S. aureus* (48%) and *S. epidermidis* (35%) predominating at the species-level.

Infected or non-infected?

Of the studies employing amplification and sequence analysis of the 16S rRNA gene to characterize the microorganisms involved in DFI, few have sampled participants with overt clinical signs of infection. Expert consensus opinion is that clinically uninfected wounds should not

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be treated with antimicrobials and therefore should not be cultured [3,24]. The clinical relevance of culturing uninfected DFUs to characterize the microbiome is debatable. In patients with diabetes presenting with overt clinical signs of infection, the decision to obtain a culture from a wound to identify a pathogen of infection is clinically justified. Many patients with diabetes, however, do not display overt clinical signs of infection in the presence of a non-healing DFU [25,26].

Some clinical researchers have proposed under these circumstances to utilize secondary signs and symptoms (i.e. malodour, increased exudate and poor quality tissue) to diagnose infections, signs that could be more pertinent to guiding action by clinicians in treating wounds that are non-healing with standard care [27]. Obtaining cultures to examine the microbiome of patients with 'masked' signs of infection in DFUs that are non-healing despite standard care may prove greatly beneficial.

Sole pathogen or polymicrobial infection?

Lastly, certain aerobic species are often overestimated as the primary pathogens in DFI. When comparing the evidence on the bacteriology of DFIs through both culture-dependent and culture-independent approaches, both corroborate *S. aureus* as being the predominant pathogen colonizing DFUs (Figure 1). In tandem with this, reports have also suggested that the abundance and role of anaerobes are greatly underestimated. This is of additional importance when defining the pathophysiology behind a DFU with previous reports documenting the increased role of anaerobes in ischemic and neuroischemic DFUs [2,10]. The significance however at a clinical level of identifying additional 'hidden' anaerobes that often form part of a polymicrobial infection are yet undefined. Many antimicrobial agents commonly used in the treatment of DFIs have a broad-spectrum of activity to cover most anaerobes. Furthermore, no published studies are available reporting improved clinical outcomes when adding additional anti-anaerobic directed therapy to concurrent regimes.

Applying metagenomics to DFI – could this be a 'game changer' in defining 'which microorganisms are present, what are they capable of doing and who did it'?

Advancements in molecular technologies have seen the emergence of massively parallel sequencing (MPS) that greatly increases the throughput of sequencing large amounts of DNA. This significantly reduces the cost to analyse clinical samples and makes MPS platforms widely applicable to use in clinical practice [28] (Figure 1). However, MPS platforms generate large and complex genomic data sets that require analysis. To complement the increasing use of MPS by both researchers and clinicians, the development of bioinformatics programs and software packages have become essential tools. Open-source software such as QIIME [29] and MOTHUR [30], for example, have been widely used in microbiome research for their ability to construct details on three areas of importance to clinicians dealing with chronic wounds: the microbial load, the microbial diversity and the presence of pathogens [31].

Unlike the targeted amplification and sequence analysis of 16S rRNA gene through PCR, metagenomics employs a complement of molecular technologies and techniques to sequence DNA extracted directly from a sample. DNA in metagenomic approaches are sheared into smaller fragments and independently sequenced in a technique commonly referred to as whole genome sequencing (WGS). This approach produces sizeable data sets requiring the alignment of DNA reads to known genes through open-access reference databases, such as NCBI GenBank [32]. When combined, these ever growing gene reference libraries enable the analysis of varying components of microbial ecology and their functions.

Applying metagenomics to DFI may allow for an extended picture of which microorganisms are there. What are they capable of doing and 'who' did it? WGS identifies all genomic DNA including bacteria, fungi, archaea and viruses (microbiome). In addition to DNA sequences involving the taxonomically informative genes, other genes of interest may be identified through WGS such as protein coding sequences from which the biological functions of the microbe can be inferred (e.g. pathogenicity (pathogenicity islands), virulence, antibiotic susceptibility [33] or metabolic pathways [34]). These protein sequences are identified through databases containing resources required for understanding high-level functions and utilities of biological systems, including known proteins for microbial cells (Kyoto Encyclopedia of Genes and Genomes – Kegg and MG-RAST).

Whole genome sequencing is however limited in its capacity to provide information on the actual metabolic activity of a microbial community, as it cannot differentiate between expressed and non-expressed genes [35]. To circumvent this limitation, metagenomic methods have been combined with other molecular approaches, such as transcriptomics and proteomics, which are capable of identifying expressed biological signatures such as RNA transcripts or proteins, respectively, that control metabolic activities in microbial communities [36,37]. In this respect, combining WGS with transcriptome or proteome analysis may afford

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the possibility to characterize not only the microbial diversity within a DFI (i.e. 'which microorganisms are present') but the functional potential (i.e. 'which microorganisms are present and what are they capable of doing and who did it') [34].

Thus, it would be highly desirable to determine which microbes are the potential 'assailants', 'co-conspirators' and 'bystanders'; this would allow for targeted antimicrobial therapy. One study exemplifying the combined use of WGS with proteomic analysis was by Lassek *et al*. who elucidated pathogen-protein expression of catheter-biofilm associated urinary tracts infections [38]. Both *Pseudomonas aeruginosa* and *Morganella morganii* were identified as the predominant microorganisms through WGS community analysis. Proteomic analysis then revealed several interesting findings at a functional level, which implicated *P. aeruginosa* as the primary driving pathogen and identified that it up-regulated proteins involved in the degradation of red blood cells, the siderophore systems for iron acquisition (i.e. bacterial growth), biofilm formation, antibiotic resistance and pathogenicity. Currently, no studies employing WGS in combination with transcriptome, proteome or metabolome data have been undertaken for DFI.

Limitations of different molecular technologies and techniques

PCR amplification of DNA fragments requires the use of primers to bind to a specific region of the 16S rRNA gene to produce a PCR product. The use of some universal primers may not be broad enough to detect the bacteria that often cause polymicrobial infections in chronic DFUs [39]. Although the hypervariable regions of the 16S rRNA gene are highly diverse, this variation is reduced between closely related taxa. This limitation can be somewhat partially offset through the use of multiple primer sets (multiplexing PCR) to amplify different hypervariable regions. Lastly, current DNA-based methods are unable to distinguish between live or dead bacteria, amplifying all DNA regardless.

Whole genome sequencing posses gargantuan and complex data sets generating up to 600 Gb of sequence data when performing sequencing runs [40]. Furthermore, both human-host DNA and microbial DNA are sequenced with approximately 90% of DNA from a sample being human [33]. Therefore, obtaining adequate coverage of microbial DNA requires deep sequencing (i.e. large data sets) with removal of human DNA sequences via computational resources.

Summary

Amplification and sequence analysis of the 16S rRNA gene for profiling the microbiome of infected and non-infected DFUs have already provided insights for the medical community by identifying that many more bacteria are present in DFUs than the corresponding view from culture-dependent methods [14]. Despite the wealth of knowledge gained from molecular microbiology, the use of PCR-based approaches have not been routinely employed in frontline clinical microbiology services for the identification of pathogens associated with infection. This limited uptake has been attributed to the low throughput and relatively high sample sequencing costs using PCR-based platforms. This may account for why it has been primarily confined as a researchers tool for characterizing the microbial profile of various infective pathologies in human health and disease.

While the progress and application of current DNA sequencing continue to rapidly evolve, thus, far we have only been enlightened with a broader view of 'which microorganisms are there'. The potential of molecular methods that employ metagenomics, transcriptomics, proteomics and metabolomics represents a potential future 'game changer' to improving clinical care in people with DFI. Their usage may help to reveal the full extent of DFI: 'which microorganisms are present, what are they capable of doing and which microorganism was responsible'? In doing so, this may allow targeted antimicrobial therapeutics.

Conflicts of interest

B. Espidido was employed as Head Scientist at the Ingham Institute during the writing of this article but has since taken a position with Illumina Biotechnology Company. All other authors have nothing to declare.

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LETTER TO THE EDITOR

Understanding the Role of Fungi in Chronic Wounds

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We thank Kalan and colleagues (1) for sharing their data on the role of fungi in chronic wounds, an area that has to date been understudied, with only one previously published article within the literature (2). Their study is therefore of great interest for clinicians in understanding the role of fungi in chronic wounds and in ascertaining whether alterations from antimicrobials to antifungals might improve outcomes.

The data presented are, however, difficult to interpret within the context of clinical management. First, Kalan and colleagues report the sampling of chronic wounds undertaken using the Levine technique with a swab. This culture method has been the subject of great debate in the diabetic foot arena, with opinions divided. Some expert groups promote tissue biopsy as the most appropriate sampling method for identifying pathogens of infection and for exploring both the microbiome and the role of biofilms (3, 6).

We note a previous study from the authors' group suggesting good concordance between culture-independent swab samples (DNA sequencing) and tissue samples (4). However, the use of swab samples from superficial tissue makes it difficult to ascertain whether any fungi identified merely resided on wound surfaces as colonizers or whether the fungi were invasive and involved deeper tissue (this may suggest a more pathogenic involvement) (5).

Second, and more importantly, Kalan and colleagues report only on the ITS1 sequences (18S rRNA) and do not include bacterial or archaeal sequences (16S rRNA). In doing so, the clinical relevance of fungi in chronic wounds becomes lost. This is because, without identifying all the microorganisms within a wound (bacterial, fungal, archaeal), one cannot determine the overall microbial load for fungi or what their relative abundances are in relation to those of other microorganisms. This allows us to understand whether a microorganism is a dominant, major, or minor player. Therefore, no assumptions can be made on the community structure, and the "mycobiome" becomes clinically uninterpretable.

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The prevalence of biofilms in chronic wounds: a systematic review and meta-analysis of published data

Abstract: The presence of biofilms in chronic non-healing wounds, has been identified through *in vitro* model and *in vivo* animal data. However, human chronic wound studies are under-represented and generally report low sample sizes. For this reason we sought to ascertain the prevalence of biofilms in human chronic wounds by undertaking a systematic review and meta-analysis. Our initial search identified 554 studies from the literature databases (Cochrane Library, Embase, Medline). After removal of duplicates, and those not meeting the requirements of inclusion, nine studies involving 185 chronic wounds met the inclusion criteria. Prevalence of biofilms in chronic wounds was 78.2 % (confidence interval [CI 61.6–89, p<0.002]). The results of our meta-analysis support our clinical assumptions that biofilms are ubiquitous in human chronic non-healing wounds.

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biofilm • systematic review • wound healing • infection • non-healing

Unlike planktonic microorganisms, biofilm phenotypes have been defined as structured consortiums of aggregated microbial cells, surrounded by a polymer matrix, that adhere to natural surfaces, to artificial surfaces or to themselves.[1] The concept of biofilms in human health and disease is now universally accepted in tuberculosis,[2] periodontal disease and dental caries,[3] cystic fibrosis,[4–6] in-dwelling medical device infections,[7] otitis media and other upper respiratory infections,[8,9] and chronic wounds.[10,11] So highly attuned are researchers to the wide involvement of biofilm-associated infections across the spectrum of human health and disease, that the US Department of Defense has recognised the significance of biofilm as being problematic in wound healing, and has prioritised research in this area.[12]

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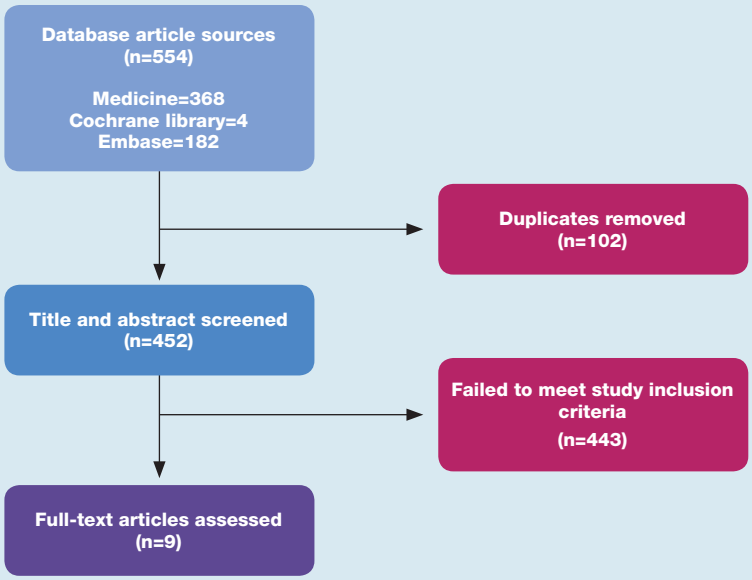
Biofilm-phenotype microorganisms residing within a chronic non-healing wound may promote a hyper-inflammatory response, detrimental to the host.[13–15] Recent observations using oxygen microsensors and transcriptomics (examining oxygen depletion in micro niches and microbial metabolic activities) have provided alternate insights suggesting that bacterial biofilm in chronic wounds may promote localised tissue hypoxia, reducing the availability of oxygen required for wound healing.[16]

Once established, biofilm often become highly tolerant to standard treatment and removal/eradication paradigms such as mechanical debridement.[17] Several features that distinguish biofilm phenotypes from their planktonic counterparts are their tolerance to antimicrobial agents,[18] disinfectants and the host's immune defences.[19,20]

While non-healing chronic wounds represent an umbrella terminology for a range of pathologies, biofilms have been cited across all related aetiologies including venous leg ulcers (VLUs),[21] pressure ulcers (PUs)[14,22] and diabetic foot ulcers (DFUs).[10] Collectively, these chronic wounds contribute to significant morbidity, mortality and increased health-care expenditure. Importantly, the continuing rise in antimicrobial resistance has placed a greater emphasis on correctly diagnosing and managing biofilm-associated infections in non-healing chronic wounds. This will require a shift in treatment paradigms to more multifaceted biofilm-based approache, given the resilience of biofilms in responding to planktonic-based treatments.

Fig 1. PRISMA flow diagram of literature search



As the presence of biofilms across the spectrum of chronic wounds has significant implications both medically and economically, clear and concise information is required to help guide health-care professionals managing these recalcitrant causes of delayed healing. An increasing body of evidence from *in vitro* models[23] and animal[24] and human studies[25] has identified the capacity of wound isolates to grow as biofilms, and for chronic non-healing wound samples to harbour biofilm.[26] This has been driven largely by advancements in molecular microbiology, microscopy technology and techniques for the study of bacterial populations *in situ*. This has allowed authors to identify biofilm as the cause of non-healing chronic wounds and in the development of associated clinical infections.

The bulk of evidence supporting the notion that biofilms complicate non-healing chronic wounds is from *in vitro* model and *in vivo* animal data.[27–30] A recent review of the scientific literature for the presence of biofilms in chronic wounds has eloquently explored the models used.[31] However, human chronic wound studies are under represented, with most having low sample sizes. For this reason, we aim to determine the prevalence of biofilms recognised in human chronic wounds by systematically reviewing the literature published on *in vivo* human chronic wound studies and to increase sample size and power by performing a meta-analysis.

Methods

Search strategy

A start date of 2008 was used based on a decision by the authors that this best represented the start of publications that used acceptable terminology and visualisation methods that best described and depicted microbial aggregates, extracellular polymeric substance (EPS) and the spatial orientation of microorganisms in samples. A systematic review of the Cochrane Library, Embase, and Medline (PubMed) databases was conducted for articles published between January 2008 and December 2015 using the following search terms: 'biofilm' [all fields] AND 'chronic wounds'.

A secondary search was undertaken using 'biofilm' with supplementary keyword filters: OR 'diabetic foot ulcers' OR 'venous leg ulcers' OR 'pressure ulcers' OR 'decubitus ulcers' OR 'non-healing surgical wounds', OR 'visualization', OR 'scanning electron microscopy' OR 'fluorescent in-situ hybridization', OR '16S rRNA'. Only articles in the English language were included. The search was limited to prospective clinical studies, case reports, case series and published conference abstracts. The systematic review was conducted in accordance with the PRISMA guidelines.[32]

Data extraction

Investigators (MM and TB) independently reviewed titles and abstracts of all articles to establish their eligibility on the basis of predefined criteria. All eligible article references were tabled and their abstracts obtained for review. Articles meeting the eligibility criteria were hand-searched for additional studies. For the purpose of the meta-analysis, we extracted the following domains or variables from the articles: date of study publication (2008–2015), prevalence rates (number of confirmed tissue samples over the total number of samples screened), sample size and study design.

Study eligibility

Articles publishing data on *in vivo* human chronic wounds, in participants over the age of 18, were included. Chronic wound aetiologies included in the search were DFUs, VLUs, PUs and non-healing surgical wounds (NHSWs). Individual searches of the methodology section were undertaken and universal definitions of a chronic wound or phrases denoting the chronicity of participant wounds such as 'non-healing', 'delayed healing' and/or 'chronic' were used to ensure eligibility.

Only articles detailing the presence of biofilm and bacteria in general through microscopy with or without combined molecular methods were included for review. In line with recent guidelines[33] the following visualisation techniques were deemed appropriate for the confirmation of biofilm presence: scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM), conventional and peptide nucleic acid–fluorescent *in situ* hybridisation (PNA–FISH) and microscopy with or without staining methods. Articles diagnosing biofilm presence by clinical observation were excluded. Confirmation of biofilm included all visualisations of aggregated bacteria within the wound bed.[34]

Additionally, to meet inclusion criteria, articles must have cited optimal collection methods for the

Table 1. Random–effects model of nine chronic wound biofilm studies

Author/s (year)	Study design	Number of participants	Visualisation methods	Chronic wound aetiologies	Number of samples with confirmed biofilm (%)	
James et al. (2008)[10]	Prospective study case versus control	66	Light microscopy, SEM 16S rRNA with DGGE	13 DFUs, 21 PUs 8 VLUs, 24 NHSWs	30/50	(60%)
Kirketerp-Møller et al. (2008)[39]	Prospective cohort study	22	PNA-FISH, CLSM	Unspecified chronic wounds	13/22	(60%)
Fazli et al. (2009)[41]	Prospective cohort study	9	PNA-FISH, CLSM	10 VLUs	10/10	(100%)
Thomsen et al. (2009)[44]	Prospective cohort study, sub analysis	2	PNA-FISH, 16S rRNA	2 VLUs	2/2	(100%)
Han et al. (2011)[38]	Prospective cohort study	15	PNA-FISH, CLSM 16S rRNA	4 DFUs, 5 PUs, 2 VLUs 4 NHSWs	9 /15	(60%)
Neut et al. (2011)[43]	Case report	2	CLSM	2 DFUs	2 /2	(100%)
Oates et al. (2014)[40]	Prospective cohort study, sub analysis	4	FISH, SEM	4 DFUs	4 /4	(100%)
Martinez-Velasco et al. (2014)[36]	Prospective cohort study, conference abstract	20	SEM, LM	Unspecified chronic wounds	20/20	(100%)
Honorato-Sampaio et al. (2014)[37]	Prospective cohort study	45	TEM	45 VLUs	45/45	(100%)

SEM–scanning electron microscopy; 16S rRNA–16S ribosomal RNA sequencing for identification of microbes; DGGE–denaturing gradient gel electrophoresis; PNA–FISH–peptide nucleic acid–fluorescent *in situ* hybridisation, CLSM–confocal laser scanning microscopy; LM–light microscopy; TEM– transmission electron microscopy; DFU–diabetic foot ulcer; PU–pressure ulcer; VLU–venous leg ulcer; NHSW–non-healing surgical wound

sampling of chronic wounds with tissue biopsy, curettage or debridement material being regarded as gold standard. Swab cultures of the wound bed were excluded for being inadequate for biofilm identification, given the inability to distinguish between planktonic and biofilm phenotype.[33]

Statistical analysis

Data from studies were extracted as raw numbers using the number of samples with confirmed biofilm over the total number of samples obtained. Data were analysed using comprehensive meta-analysis software (Biostat Inc., NJ, US). Prevalence estimate rates, weighted averages and 95% confidence intervals (CIs) were undertaken using fixed-effects meta-analysis. Forest plots were reported for inconsistencies in effect sizes and their CIs. Between-study variance or heterogeneity in estimates was modelled using Cochran's Q and the I^2 statistic. Where Cochran's Q value was reported with p-values less than 0.10 and I^2 values exceeded 50 %, a random-effects model was used.[35]

Results

Search results

The search identified 554 studies from the literature databases. After removal of duplicates, exclusion and the screening of 452 titles and abstracts, eight studies involving 185 chronic wounds met the inclusion criteria (Fig 1). The numbers of each respective chronic wound were: DFUs (n=33), VLUs (n=67), PUs (n=26), NHSWs (n=28), unspecified chronic wounds (n=31). There were eight articles from prospective cohort studies with the remaining one study being case reports/series (Table 2). Primary authors were contacted for data from two studies in order to clarify the number of positive biofilm samples.[36,37] Between-study results identified heterogeneity (Q test $p<0.022$, $I^2=55\%$); to address this, a random-effects model was used with prevalence rates reported.

Prevalence of biofilms in chronic wounds

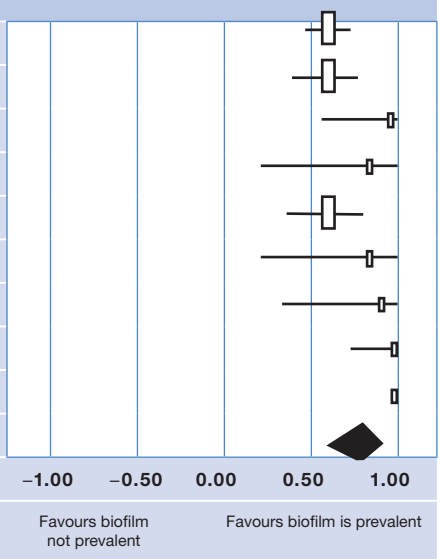
The prevalence of biofilms in chronic wounds was 78.2 % [CI 61.6–89, $p<0.002$]. Biofilm prevalence across studies identified the percentage(s) of positive biofilm samples was no lower than 60 % (noted in three studies),[10,38,39] with all remaining studies identifying 100% biofilm prevalence.[36,37,40–43] Given the relatively small sample size and the covariable of four different chronic wound aetiologies, inferences regarding whether biofilms were more prevalent in one particular chronic wound were not possible.

Discussion

Early publications providing evidence for the presence of biofilm in chronic wounds have provided guidance for clinicians and researchers alike.[10,11,39] These studies

Table 2. Random–effects model of nine chronic wound biofilm studies

Study authors (year)	Statistics for each study					Event rate and 95% confidence interval
	Event rate	Lower limit	Upper limit	Z-value	p-value	
James et al. (2008)[10]	0.600	0.460	0.725	1.405	0.160	
Kirketerp-Moller et al. (2008)[39]	0.591	0.382	0.772	0.848	0.396	
Fazli et al. (2009)[42]	0.955	0.552	0.997	2.103	0.035	
Thomsen et al. (2009)[44]	0.833	0.194	0.990	1.039	0.299	
Han et al. (2011)[38]	0.600	0.348	0.808	0.769	0.442	
Nuet et al. (2011)[43]	0.833	0.194	0.990	1.039	0.299	
Oates et al. (2014)[40]	0.900	0.326	0.994	1.474	0.140	
Martinez-Velasco et al. (2014)[36]	0.976	0.713	0.999	2.594	0.009	
Honorato-Samaio et al. (2014)[37]	0.989	0.849	0.999	3.172	0.002	
Total	**0.782**	**0.616**	**0.890**	**3.102**	**0.002**	



identified that biofilms were present in 60% of non-healing chronic wounds. Since then, studies employing combined molecular and microscopy methods to directly visualise biofilms have gathered pace.

This systematic review and meta-analysis has collated all available *in vivo* studies pertaining to the identification of biofilms from non-healing human chronic wounds. In doing so, our meta-analysis results suggest that biofilms are prevalent in all these wounds. Prevalence rates identify that 78% of non-healing chronic wounds harbour biofilms, with prevalence rates varying between 60% and 100%. We propose, therefore, that biofilms are ubiquitous in nearly all non-healing chronic wounds and the disparity in prevalence rates may be a reflection of study design and methodological limitations. For example, we argue that heterogeneous distribution of microorganisms within wounds may allow for variability in sampling, increasing the likelihood of returning negative or inconclusive samples.

Previous studies,[39,44,45] have highlighted the heterogeneous spatial distribution of wound microbiota through sampling multiple areas of the wound bed, identifying vast shifts in community diversity. This suggests that relying on a single site for sampling may reduce the chances of visualising biofilm. Obtaining samples from multiple sites of the wound may improve the detection of biofilm. However, this is often not feasible at a clinical level and is reflected in many studies that employ tissue collection methods.

The primary aim of this systematic review and meta-analysis was to provide a statistical approach for further justifying the evidence that biofilms are present in chronic non-healing wounds. We acknowledge that our analysis has obvious limitations, in particular the low number of human studies available and the requirement for further *in vivo* studies with larger sample sizes to support existing data. This, however, further emphasises our rationale of performing a meta-analytical approach.

It was also not our intention to provide guidance for treatment of chronic wounds; for that we would like to refer to the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guideline for the diagnosis and treatment of biofilm infections.[33]

Another limitation or difficulty with analysing the presence of biofilm in chronic wounds has centred around 'what we define as a biofilm'. Often, biofilms are defined based on *in vitro* observations, and these describe biofilms as bacteria attached to surfaces within a self-produced extracellular matrix and tolerant to antimicrobials. In addition, biofilm development is often described over three to five stages, initiated by planktonic bacteria attaching to a surface, maturation of the biofilm and, lastly, dispersal of bacteria from the biofilm.[46]

However, *in vitro* observations based on flow cell models using glass surfaces and fresh oxygenated culture media continuously flowing over the bacterium, differ greatly from the conditions within chronic wound infections.[34] Here, the bacteria are not exposed to a continuous flow of fresh media and are not attached to a glass surface (or to a surface at all).[10,39] *In vivo* chronic wound biofilms are also often encapsulated in a matrix, which includes host material, making dispersal problematic.

Therefore, using *in vitro* observations to define, diagnose and treat biofilm in chronic infections may provide a misguided impression.[47] There are, however,

commonalities between *in vitro* and *in vivo* evidence that can help in providing a definition of a biofilm. These include: aggregation of bacteria, some sort of matrix that is not restricted to self-produced as it can also be of host origin, and extreme tolerance and protection against most antimicrobial agents and the host defence.

We suggest following this simplified definition in order to define biofilm in chronic infections: an aggregate of bacteria tolerant to treatment and the host defence.

Conclusion

When combining the results of our systematic review and meta-analysis with the available *in vitro* models and animal studies it seems highly likely that biofilms are ubiquitous in non-healing human chronic wounds. It is important that clinicians appreciate the distinct differences of biofilm phenotypes to their planktonic counterparts, in particular the challenges in eradicating and removing biofilm from a wound. A paradigm shift to a biofilm-based wound care approach should be adopted. **JWC**

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APPENDIX 5

STUDY PROTOCOL SUBMITTED TO SOUTH WESTERN SYDNEY LHD ETHICS AND RESEARCH COMMITTEE

TITLE

The Microbiome of chronic wounds

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Protocol Version: 1.0

Date: 12/08/2014

Sponsor:

South West Sydney Local Health District

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The Microbiota of Chronic wounds

1. Purpose of Proposed Investigation

Research into chronic wound bacteria has traditionally focused on planktonic cells, however bacteria encountered in nature and medical diseases commonly function as multispecies communities that communicate, live and work together in a symbiotic and or pathogenic manner (microbiota).

This multispecies community can advance to encase itself in a protective extracellular polymeric substance and attach to a living or non-living surface, and this has been commonly described as a biofilm [144]. More than 99% of bacteria found in nature exist in these stable, persistent biofilms, and there are reasons to believe this bacterial theme also holds true in the chronic wound environment.

In recent years electron microscopy of biopsies from chronic wounds have found that 60% of the specimens contained biofilm structures in comparison to only 6% of biopsies from acute wounds [12]. The significance of the bacterial diversity and any symbiotic and pathogenic relationships between bacterial organisms in addition to the aetiological role of biofilms in causing chronic wound infection remains poorly understood. Currently culture based techniques to determine the bacterial organisms residing in chronic wounds select for species which flourish under the typical nutritional and physiological conditions of the diagnostic microbiology laboratory and this may not necessarily reflect the most abundant or clinically important organisms in chronic wounds [10].

An observational study is therefore proposed using advanced molecular techniques (Pyrosequencing), confocal laser scanning microscopy (CLSM) and labelled DNA probes for the in situ identification of microorganisms (FISH) in exploring the bacterial diversity (microbiota) that reside in chronic wounds. Additional primary aims are to explore if biofilms are present within chronic wounds and if so are they mainly comprised of planktonic or multiple species of bacterial organisms. Our secondary aims are to correlate clinical wound parameters with the positive presence of biofilm.

We also plan to undertake a separate pilot study testing the effectiveness of two antimicrobial woundcare treatments in reducing bacterial numbers and biofilm formation within chronic wounds *in vivo*. An analysis of biofilm structure will be utilized to determine effect using the parameters; species composition and the total number of bacteria.

2. Background

Chronic wounds including diabetic foot ulcers (DFU) represent a significant cost in both financial and human terms. This cost is sure to rise as the prevalence of diabetes and obesity increases with a concomitant increase in the incidence of chronic wounds. Four interlinked factors are responsible for contributing to chronic wounds. These are local tissue hypoxia, bacterial colonisation/infection, repetitive ischaemia-reperfusion injury and general patient aging. Each component of the pathogenesis is deleterious in isolation but together they can overwhelm the healing response in many patients. Of these, the only factor that is readily treatable in all wounds is bacterial infection.

The role of bacteria in chronic wounds

All chronic wounds contain bacteria. The presence of bacteria in the wound does not, however, indicate infection or a barrier to wound healing. The impact of bacterial organisms on healing will depend on the pathogenicity of the organism, the bacterial load and diversity, the toxin produced and the host response. Extensive research into the role of bacteria in causing chronic wound infections has identified contrasting evidence. In particular, the focus has centred on the roles of single, free-floating bacteria (planktonic) and or the interaction of multiple species of bacteria in causing infection. In some chronic wounds one or more species of organisms may proliferate in the wound and it is this ecological community of commensal, symbiotic, and pathogenic microorganisms that constitute the "microbiota"

The microbiota in some chronic wounds may additionally form a highly organized, dynamic heterogenous community of organisms that has been termed a 'biofilm'. At the most basic level a biofilm has been described as a "microbial population that has attached to a biological or non-biological surface and in an attempt to flourish this community embeds itself in a protective extracellular polymeric substance (slimy barrier of sugars and proteins) [93].

Once the formation of a biofilm exists in a chronic wound the opportunity of this community to attack the host tissue may lead to tissue damage and a host response that is infection. Whilst bacterial colonization and infection play an integral role in chronic wounds the exact role of biofilms has remained unclear. In recent years' electron microscopy of biopsies from chronic wounds have found that 60% of the specimens contained biofilm structures in comparison to only 6% of biopsies from acute wounds [12,141]. The role of biofilms has now been well documented as medical problems associated with implants and certain other diseases.

However, the nature and importance of wound biofilms is only now beginning to be realized as reviewed and discussed in the scientific literature [12]. Chronic wounds, including diabetic foot ulcers (DFUs), venous leg ulcers (VLUs), and pressure injuries (PIs), are often resistant to natural healing and require long term medical care and are also implicated as a major source of both morbidity, mortality that carry extensive healthcare and quality of life costs.

Methods of identifying bacterial organisms

Traditionally, to detect potential pathogens of infections, wound cultures have been utilised to identify free floating (planktonic) organisms in addition to looking at their density through qualitative and quantitative measures. The limitations of such are that culture-based techniques select for species that flourish under the typical conditions of the diagnostic microbiology lab and this may not necessarily reflect the most abundant or clinically important organisms in chronic wound infections. It has also been recognised that bacterial organisms within biofilms cannot be detected using normal wound culture methods [13].

More recently molecular based microbiological techniques have raised doubts about the role of traditional culturing techniques and may eliminate the bias open to traditional methods. Using amplification and sequence analysis of 16s rRNA subunit, a highly-conserved gene present in all prokaryotes (bacteria) but not eukaryotes (humans) that contains hypervariable regions allowing for bacterial identification, has revealed a vastly more complex array of bacterial communities than those identified by traditional culture methods in chronic wounds [152-155].

This presents an important problem and discrepancy because many of the bacteria in wound biofilms are recalcitrant to culture. Thus, there is a lack of information about the diversity of populations that occur in association with chronic wound pathogenic biofilms. A better

understanding of bacterial populations associated with wound biofilms is necessary to enable development of next generation management and therapeutics.

Methods of visualizing bacteria

Bacterial biofilms are encased within an extracellular polymeric substance (EPS) of which the bacteria themselves produce. It has been generally accepted within the medical community that biofilms are not visible to the naked eye. However, EPS has been visualized by scanning electron microscopy (SEM), whilst Individual bacteria and bacterial microcolonies have also been observed using fluorescence in situ hybridization (FISH) on chronic wound biopsy sections, which permits the visualization and identification of individual bacteria in human disease states in situ [93].

The infection continuum in chronic wounds

The concept of the wound infection continuum model places an emphasis on progression from colonization of bacteria within a wound through to infection, with infection being dependent on an increase in microbial load. An important component of the wound continuum model is the concept of “critical colonization”. Critical colonization is defined as “multiplication of organisms without invasion but interfering with wound healing. Whilst this concept of critical colonization is still the center of much debate its concept has been used by clinicians to explain an alteration in the wound bioburden associated with delayed wound healing in the absence of clinical infection. This concept is important for clinicians as chronic wounds with critical colonization may benefit from local and or topical treatments such as antimicrobial wound dressings and wound debridement, rather than systemic management with the use of antibiotics.

Clinical Methods of identifying infection and biofilm in Chronic Wounds

Identification of Infection in chronic wounds such as DFU's is imperative to prevent complications such as amputation. In persons with diabetes it is thought that over 90% of Infections are the primary pathway to lower extremity amputation. Whilst there has been much debate as to the optimal methods to diagnose infection, the Infectious Disease Society of America have recommended a specific combination of clinical signs and symptoms to aid diagnosis in their consensus guidelines on diagnosing diabetic foot infections [2]. Underpinning the guidelines are the recommendations that the presence of infection be based on the presence of purulent exudate or two or more signs if inflammation (pain, erythema, heat or oedema).

In contrast to the above are reports from other groups who have highlighted that clinical signs of infection may however, not always present in some people with chronic wounds, with the classic signs of infection being retarded by confounding variables such as the presence of peripheral neuropathy, peripheral arterial disease, hyperglycaemia and established biofilm phenotypes.

Wound Infection is the host response to tissue injury with an inflammatory response being the first line of defence against bacterial invasion. It has been reported however that many chronic wounds do not express these signs of clinical infection despite high microbial loads and or the presence of pathogenic organisms. For this reason Gardner and colleagues proposed a second guideline to follow in cases of suspected infection in a chronic wound where the 'classic' clinical signs maybe absent [19]. The clinical signs and symptoms checklist (CSSC) objectively measures clinical signs of infection in chronic wounds and is a linear combination of 12 separate signs of infection.

It is generally regarded the biofilm is not visible to the naked eye and there is significant debate as to whether clinicians can rely on clinical indicators to determine the presence of a biofilm in a chronic wound. In a recent publication by Keast *et al* 2014, a table of clinical indicators that may present in the presence of chronic wound biofilm were proposed based on supporting evidence [432].

Overview of the current research - gaps in the evidence

Little is currently known about the types of bacteria that might contribute to the bacterial diversity in infected chronic wounds. More importantly there is a large gap in the available evidence on what and which bacteria play important roles in chronic wound infections and if these infections are caused by free floating planktonic pathogens or biofilm phenotypes.

In particular, there is limited evidence from researchers employing molecular based microbiology and of these only a few recent surveys of bacterial populations associated with various chronic wounds including diabetic foot ulcers are available within the literature. Most have focused on the role of easily cultured and well-known pathogens such as Staphylococcus aureus and Pseudomonas aeruginosa. These organisms are cultured easily using traditional microbiological evaluations, and, therefore, standard non-molecular methods likely overestimate the contribution of these species to the microbiota of chronic wounds.

Of the available molecular based studies on the microbiota of chronic wounds and DFUs, few have sampled from participants with clinical signs of infection. Whilst the standard treatment for diabetic foot infection and chronic wound infection includes but is not limited to the use of systemic antimicrobial therapies the clinical significance of sampling clinically uninfected DFU's remains unclear.

Other studies within the context of chronic wound microbiota sampled wounds that were heterogeneous in aetiology. Gardner and colleagues 2013, undertook one of the only available studies on DFU microbiota and proposed that the sampling of DFU's should be from a homogenous DFU aetiology group [10]. For this reason, Gardner and colleagues sampled the microbiota of neuropathic ulcers, however this does not reflect the typical patterns of presentation in the diabetes foot population, with neuroischemic DFU's forming most clinical presentations.

The microbiota of chronic wounds from a molecular perspective remains largely ambiguous. Understanding the roles differing organisms play and the role of biofilm phenotypes in causing infection in chronic wounds would be highly significant both economically and medically and may allow for target specific treatment modalities.

3. Study Rationale

The significance of the bacterial diversity and any symbiotic and pathogenic relationships between bacterial organisms in addition to the aetiological role of biofilms in causing infections in chronic wounds remains poorly understood.

We propose to explore the bacterial diversity in infected wounds (microbiota) by comparing results from standard lab-based culture against advanced molecular techniques (PCR), Confocal Laser Scanning Microscopy (CLSM) and labelled DNA probes for the in situ identification of microorganisms (FISH). A better understanding of bacterial populations and the role of biofilms associated with chronic wound infection is necessary to enable the development of next generation management and therapeutics.

Secondly, it has been recognised within the literature that bacterial organisms within biofilms cannot be detected using normal wound culture methods and wound biofilm are not visible to the naked eye. We propose to correlate any relevant clinical wound parameters with microscopy results for positive biofilm presence against previously suggested clinical indicators of biofilm presence in chronic wounds. This may allow for the development of a clinical based tool based on signs and symptoms of possible biofilm presence that may aid clinicians in a timely diagnosis and the potential to alter treatment regimes.

Thirdly, we propose to undertake a pilot study to determine the effectiveness of two commonly used antimicrobial woundcare treatments on reducing bacterial numbers and biofilm formation over a 7-day period *in vivo*. Chronic wounds with suspected high bacterial loads (critical colonisation) or biofilm formation are commonly treated with various antiseptic or antimicrobial wound dressings and or ointments that have purported mechanisms of action to aid wound healing by reducing the amount of bacteria present in the wound. Most works undertaken in this arena are from *in vitro* sources.

Disappointingly, these wound care treatments often have variable results with some chronic wounds progressing to a more serious infection and possibly amputation. One potential explanation for this treatment failure is because the wound care treatments do not target or are ineffective against biofilm. If successful this pilot study may improve patient care and pave the way for future studies in this developing area. Given the financial implications of utilising new advanced molecular and microscopy techniques we plan to undertake this pilot study only if we can secure funding from external sources.

Statistical Power

Phase I will be the initial observational study of 30 patients with chronic wound infection and will be self-funded.

Contingent on obtaining adequate funding, Phase II aims to recruit sufficient patients to provide a robust estimate of the required sample size for a larger trial. An additional 30 patients (15 per cohort) will be recruited as part of Phase II.

We estimate that n=30 patients will accommodate a 15% drop out rate while retaining sufficient statistical power to estimate the effect size and variance of the effects of each wound treatment.

4. Study design

This study has two clear phases. The first phase is to undertake an observational study of 30 participants exploring the diversity and relationships of bacterial organisms within chronic wounds. The second phase is a pilot study of 30 participants designed to estimate the effectiveness of two commonly used woundcare treatments in reducing bacterial burden in chronic wounds *in vivo*.

4.1 Primary and Secondary aim/s of Cohort 1

4.1.1 Primary aim/s

- To explore what bacterial organisms commonly reside in infected and non-infected chronic wounds.

- To determine if biofilms are present in chronic wounds and if they are principally composed of single or multiple species of bacteria.

- If biofilms are identified, is there a common bacterial organism responsible for creating the biofilm.

- To compare lab-based culture against PCR, SEM and FISH.

4.1.2 Secondary aim/s

- Correlate clinical indicators to the presence of biofilm.

4.1.3 Primary aim of cohorts 2 and 3

1. Assess the effectiveness of an antimicrobial woundcare treatment in reducing bacterial numbers and biofilm formation *in vivo*. An analysis of biofilm structure will be utilized to determine effect using the parameters; species composition and the total number of bacteria.

5. Methodology

Phase I

A convenience sample of 30 participants presenting with chronic wound infection will be enrolled from Liverpool Hospital High Risk Foot Service over a 12-month recruitment period.

Cohort one: will consist of 30 chronic wounds (DFU's, venous leg ulceration, pressure ulceration) with clinical signs of infection, which are located on the lower extremity. One tissue biopsy sample measuring 3mm will be obtained from the wound edge for each participant at initial presentation.

Phase II

A convenience sample of 30 participants presenting with a chronic wound and signs of high bioburden but no clinical infection will be enrolled from Liverpool Hospital High Risk Foot Service clinic over a 12-month recruitment period.

Cohorts two and three will consist of 15 chronic wounds in each cohort with no clinical infection but signs of a high bioburden (critical colonization) that maybe responsible for the delayed healing of the wound. In these cohorts, it is planned to assess the effectiveness in reducing chronic wound biofilm over a 7- day period.

Cohort two: An antimicrobial wound irrigation solution and antimicrobial hydrogel (Wound aid® wash, and woundaid® hydrogel, Rye Pharmaceuticals) will be applied daily for 7 days. One tissue biopsy sample measuring 2mm will be obtained from the wound edge for each participant at initial presentation. An additional 2mm tissue biopsy for this cohort will be obtained at day 7 following the consecutive daily application of the products. A smaller biopsy size of 2mm will be used for cohort 2 as we do not plan to send for routine laboratory culture and as such less tissue is required for analysis.

Cohort three: An antimicrobial ointment (Cadeoxmer Iodoine, Iodosorb®, Smith and Nephew) will be applied twice over the 7-day period, at initial presentation and at day 4. One tissue biopsy sample measuring 2mm will be obtained from the wound edge for each participant at initial presentation. An additional 2mm tissue biopsy for this cohort will be obtained at day 7. A smaller biopsy size of 2mm will be used for cohort 2 as we do not plan to send for routine laboratory culture and as such less tissue is required for analysis.

In addition to the above all participants will undergo a wound assessment at each visit in keeping with standard care.

Analysis of tissue

Tissue biopsies for participants in cohort 1 will be subjected to routine laboratory culture-based analysis and advanced molecular microbiological and microscopy techniques, with routine laboratory culture-based analysis being omitted for cohort 2. Samples will be processed for;

• Amplification and sequence analysis of 16s rDNA (bacteria) and 18s rDNA (human). This is necessary to determine all the bacterial species in the wound biopsy in addition to performing qPCR to obtain the total number of bacteria per mg of tissue expressed as a percentage.

• Bacterial aggregates will be detected and located by fluorescence in situ hybridization (FISH) using fluorescent labeled bacterial universal probes and bacterial species-specific probes. Bacteria are detected with 16S rDNA fluorescein labeled EUB537 universal probe that detects all species of bacteria.

• Routine laboratory culture-based analysis for microbiology, culture and sensitivity that will be used first and foremost by the high-risk foot team to guide any treatment therapeutics and or management plans for the newly diagnosed chronic wound infection. The quantitative analysis section of the tissue sample will also help to determine species and bacterial density that will be used as the comparison against FISH, CLSM and PCR.

- Confocal Laser Scanning Microscopy to determine the presence of biofilm will be undertaken in selected participants. This will be dependent upon the ability to obtain an additional 1mm tissue biopsy sample.

It is also intended to collect additional clinical data which forms part of routine care;

- Broad demographic data
- Broad clinical data including wound size, location of the ulcer, ulcer etiology, University of Texas ulcer grade classification.
- Broad laboratory data including FBC; WCC, ESR, CRP, HbA1C

All participants will continue to receive standard care over the study period, of which the treatment for the chronic wound will be dependent upon the medical requirements of each study participant. Additionally, the collection of tissue biopsies from people with suspected infection and high bioburden form part of gold standard care and is the preferred culturing technique to identify pathogens of infection. The technique of obtaining tissue biopsies from the wound edge in chronic wounds have shown rapid healing and do not result in delayed overall healing of the wound.

5.1 Inclusion criteria

- Type 1 or type 2 diabetes mellitus
- Chronic wound >6 weeks duration (Neuroischemic Diabetic Foot Ulceration, Venous Ulceration, Pressure Ulceration). Neuroischemic DFU is defined as;

1. A modified neuropathic disability score >6 and ABI>0.5 / in-palpable pedal pulses with Doppler waveform of bi-phasic and above and or confirmed vascular disease in the medical history with further evidence through vascular diagnostic imaging.
2. A University of Texas wound classification of 1B and 2B.

- Clinical signs of infection as per IDSA guidelines or masked signs of infection as per the clinical signs and symptoms checklist (CSSC).

- ABI >0.5

- No known osteomyelitis

5.2 Exclusion criteria for cohort 1

- No oral, topical or systemic antimicrobial therapy 2 weeks prior to enrolment

- Current anticoagulation therapy such as warfarin, clopidogrel and INR>2.0

5.3 Exclusion criteria for cohorts 2 and 3

• A change in current antibiotic regime within 2 weeks prior to enrolment and or during the treatment period.

- Current anticoagulation therapy such as warfarin, clopidogrel and INR>2.0

5.4 Identification of Infection

5.4.1 Clinical signs of infection

The Infectious Disease Society of America Guidelines 2012 will be used to identify clinical infection [2].

5.4.2 Secondary signs of infection

Secondary signs of infection in chronic wounds have been proposed by Gardner and colleagues (2009) [19] and will be used to identify potential chronic wounds with masked signs of infection.

5.4.3 Identification of clinical parameters to determine the presence of wound biofilm

Clinical parameters that maybe associated with wound biofilm have been proposed by Keast et al (2014) [432]. The clinical parameters will be collected for each participant at each visit.

5.4.4 Identifying wounds with high bioburden / critical colonisation BUT not infection

Local signs of delayed wound healing due to possible high bioburden have been proposed when all other treatment modalities are in place; peri-wound erythema, pain, odour, oedema, or heavy exudate.

5.5 Antimicrobial wound Irrigation solution (Woundaid® wound wash, TGA number AUST L 221556)

In Australia, Woundaid® wound wash has been approved by the Therapeutic Goods Administration (TGA, Wound Wash TGA registered AUST L 221556) for the use of irrigation, moistening the wound bed, removing devitalised tissue and managing bioburden from;

- Acute Wounds – cuts, abrasions, lacerations
- Chronic Wounds – diabetic, pressure & venous ulcers, abscesses
- Entry Ports & Peristomal Skin;

- For intraoperative cleansing of wounds, during negative pressure closure, and to moisturise gauzes, bandages and other physical dressings during dressing changes

The active antimicrobial agent is 4% tea tree oil with the surfactant PEG-35 castor oil to aid in the reduction of surface tension. The Woundaid wash has fully solubilised Melaleuca Oil within the liquid. This enables the Melaleuca Oil to be more quickly bio-available – hence better suited to lower contact time wash. Furthermore, the increased surfactant aids reduction of surface tension – with clinical studies indicating benefits in breaking down bio-films.

5.6 Antimicrobial wound hydrogel (Woundaid® TGA registered AUST L 114360)

In Australia, Woundaid® hydrogel has been approved by the Therapeutic Goods Administration for the use of moistening the wound bed, removing devitalised tissue and managing bioburden from;

- Acute Wounds – cuts, abrasions, lacerations
- Chronic Wounds – diabetic, pressure & venous ulcers, abscesses
- Entry Ports & Peristomal Skin;
- For intraoperative cleansing of wounds, during negative pressure closure, and to moisturise gauzes, bandages and other physical dressings during dressing changes.

The active antimicrobial agent is 4% tea tree oil with a sustained, slow release action to complement the pre-application of woundwash. The surfactant Polysorbate 20, is to aid solubilisation. The surfactant used in Woundaid Wound Hydrogel is Tween 20 – a non-ionic surfactant. The surfactant concentration is a much lower level in Woundaid Hydrogel than in Woundaid wash. In the hydrogel, the Melaleuca Oil is physically trapped in the gel and not fully solubilised in the system, leading to a slower rate of bio-availability of the Melaleuca Oil

in the wound – hence a slower release over a period of time due physical contact and changes in the gel structure.

5.7 Antimicrobial Cadexomer Iodine (Iodosorb® Ointment TGA registered AUST R 43364)

Iodosorb® has been approved by the Therapeutic Goods Administration as an antimicrobial medicated ointment used in various types of chronic or acute wounds. Iodosorb® reduces the bacterial count, facilitates de-sloughing, absorbs exudate and maintains a moist wound environment to promote healing of chronic skin ulcers. Iodine is physically immobilised within the matrix of the dry cadexomer iodine and is slowly released in an active form during uptake of wound fluid. This mechanism of release provides antibacterial activity both at the wound surface and within the formed gel.

5.8 Specimen collection

5.8.1 Tissue biopsy

A 2mm or 3mm punch biopsy will be obtained from the wound edge of each chronic wound. A clinician with a competency in tissue biopsy technique will obtain biopsy samples following a standard punch biopsy method.

5.8.2 The use of local anaesthesia

We envisage that a high percentage of the patient population who present with chronic wounds whom are eligible for this study will have a peripheral neuropathy through underlying disease processes such as diabetes mellitus. In these circumstances, it is unlikely that a local anaesthesia will be required prior to undertaking the tissue biopsy procedure. In participants who do not have a peripheral neuropathy, a local anaesthesia via sub-cutaneous injection will

be required. Other clinicians at enrolled sites will administer their local anaesthesia in keeping with their own competency and local guidelines.

5.8.3 Possible complications from tissue biopsy

Complications are uncommon following this simple procedure but all medical procedures carry a potential risk. Possible complications following a tissue biopsy include:

- Excessive bleeding at the site of biopsy (haemorrhage)
- Infection
- Puncture damage to nearby tissue or organs such as arterial vessels
- Skin numbness around the biopsy site

5.8.4 Minimization of risk for tissue biopsy

It is expected that some bleeding will occur during the biopsy procedure. In most cases this can be controlled by applying local pressure and or a haemostatic dressing if required. To minimize the risk of excessive bleeding anyone on anticoagulation therapy such as warfarin or clopidogrel will be excluded from this study.

To minimize infection all procedures will be undertaken in an aseptic manner and in keeping with the tissue biopsy protocol. In cohort 1 we already suspect the presence of underlying infection and therefore the risk of further infection from a biopsy procedure is minimal and outweighed by the requirements to correctly identify the potential pathogen/s of the already suspected infection.

Puncture damage to potential vessels will minimized by following biopsy protocols. Skin numbness may not present a problem for a high percentage of participants who will already

have underlying peripheral neuropathy, however adherence to the correct technique as outlined previously will help limit potential complications.

In all cases the size of tissue biopsy has been set at the lowest size possible to obtain an adequate analysis for all tests. No single tissue biopsy size is greater than 3mm in dimension and as such the biopsy site/s will not require suturing to close. For cohort 1 a 3mm tissue biopsy is required for testing PCR, FISH and lab-based culture. For Cohort 2 a 2mm tissue biopsy is sufficient to run analyses on just PCR and FISH.

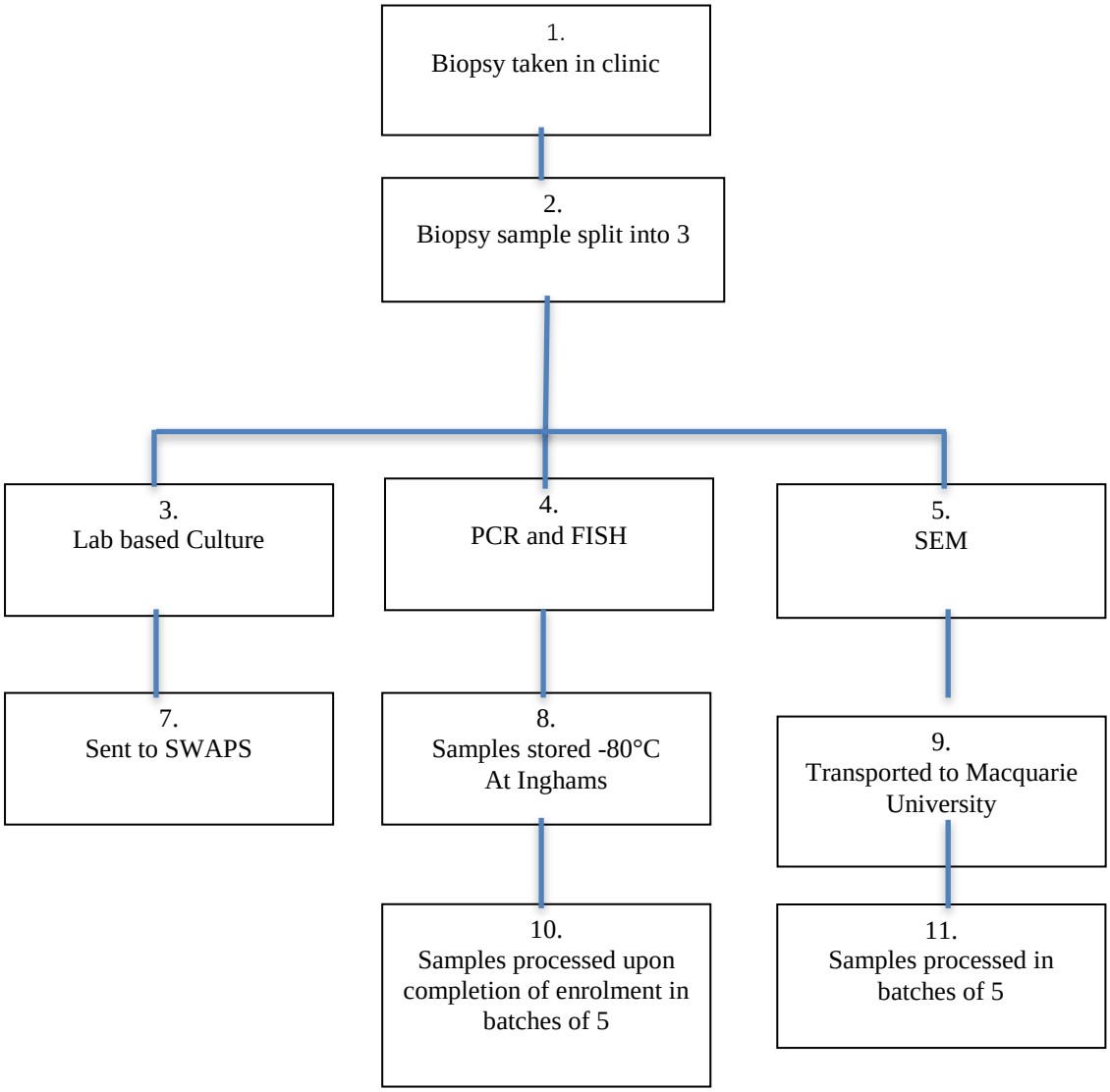
For CLSM an adequate size of tissue would not be possible from undertaking just one biopsy sample. Therefore, an additional tissue sample of 1mm (the lowest of all tissue punch biopsies available) is required and this is additional to standard care. We will ensure all participants are appropriately aware of this prior to consent. Having to seek this additional tissue sample is in keeping with previous published methodologies. The protocol for deciding to take an additional 1mm tissue sample for CLSM will be if the chronic wound is >40mm in size.

5.8.5 Tissue Processing

Cohort 1 tissue samples for PCR, FISH and lab-based culture will be immediately split into three – 1mm pieces at the time of biopsy using tissue scissors in an aseptic manner. Cohort 2 tissue samples will be split into two 1.5mm pieces. One portion of tissue will be placed in a sterile jar with 30ml normal saline and transported to the microbiology department immediately for lab based quantitative microbiology, culture and sensitivity. Another sample will be placed in a sterile jar with 30ml of normal saline and transported immediately to the Ingham Institute. Here it will be placed in a microcentrifuge tube and frozen at –80◦C until study end for 16S rDNA analysis.

Lastly, a tissue sample will be placed in a sterile jar with 30 ml of saline and transported to Macquarie University where it will be embedded in optimal cutting temperature (OCT) embedding matrix and frozen at –80◦C until study end for FISH analysis. In cases where it is not possible to transport this sample immediately, samples maybe refrigerated for a maximum of 4 hours prior to transportation. Any additional 1mm tissue sample obtained for CLSM analysis will be placed in a sterile jar with normal saline and transported in a similar fashion as the FISH samples also to Macquarie University.

Tissue sampling workflow sequence.



5.9 Process of Bacterial identification, their relative abundance and biofilm identification

5.9.1 16S rDNA next generation sequencing workflow

5.9.2 DNA Extraction

5 – 10 mg of human chronic DFU biopsy samples stored in RNAlater (Qiagen) will be defrosted on ice prior to DNA extraction. Genomic DNA will be extracted using Mo Bio PowerBiofilm DNA isolation kit (Mo Bio Cat# 24000-50) following the manufacturer's instructions.

5.9.3 16S rDNA qPCR to determine bacterial load

The quantification of total bacterial DNA in each chronic wound tissue will be calculated by real-time quantitative PCR (qPCR) using universal eubacterial primer 16s rRNA_341F 5'-CCTACGGGAGGCAGCAG-3' and 16s rRNA_534R 5'-ATTACCGCGGCTGCTGG-3' to amplify a 194bp amplicon of 16s rDNA of all bacteria. Human 18s rDNA gene is used as a reference gene to normalise the amount of wound tissue used in the DNA extraction. The primer pair utilised will be 18s rRNA gene real-time PCR is 18s rRNA_756F 5'-GGTGGTGCCCTTCCGTCA-3' and 18s rRNA_877R 5'-CGATGCGGCGGCGTTATT-3' to amplify a 122bp amplicon.

Real-time PCR will be undertaken using the Mx3000P qPCR system (Agilent Technologies, CA, United States). 25µl reaction mix containing 1X Brilliant II Sybr Green qPCR Master mix (Agilent Technologies, CA, United States), 400nM forward and reverse primers, and 100ng DNA template, will be subjected to the following cycling conditions: activation of Taq polymerase at 95oC for 10 min, followed by 40 cycles of denaturation at 95oC for 15 seconds, annealing at 56oC for 30 sec and extension at 72oC for 30 sec.

Each qPCR run is undertaken with standard samples of known concentrations (copies/µl). Ten-fold serial dilutions of the quantified 16s rDNA, and ten-fold serial dilutions of 18s rDNA PCR

amplicon solution, kept in aliquots at -20oC are used as external standards of known concentration (copies/µl) in real-time PCR reaction. The standard samples are ranged between 102–106 copies/µl, which is used to construct a standard curve for each qPCR run. The calibration curve is created by plotting the threshold cycle (Ct) corresponding to each standard vs the value of their corresponding gene concentration (copies/µl). The copy number of 16S rDNA (copies/µl) is normalised against the copy number of human 18s rDNA (copies/µl) in each chronic wound sample.

Calculations of total bacteria number per mg tissue based on qPCR 16S rDNA copy number Convert 16S rDNA copy number to total bacterial number was based on average of 5 copies of 16S rDNA per bacterial cell. Direct conversion: total bacterial number per mg tissue = 16S rDNA copy number (copies/µl) x DNA elution volume 100 (µl) = Total 16S rDNA copy number from extracted tissue. Total 16S rDNA copy number extracted from tissue / weight of tissue (5 mg) = 16S rDNA copy number per mg tissue. 16S rDNA copy number per mg tissue / 5 = total bacteria number per mg tissue.

Indirect conversion normalises to human 18S rDNA: total bacterial number per mg tissue. This is more accurate independent to DNA extraction yield. Human genome size is average 3.2 Gb base pair and each human genome contains 225 copy number of 18S rDNA. Molecular weight of each human genome DNA is 3.2 x 10^9 X 660 = 2.112 x 10^12. One mole (6.023 X 10^23 copy) of human genome DNA would be 2.112 x 10^12 g, or 2.112 x 10^18 µg. One µg of human genome DNA would have 6.023 x 10^5 / 2.112 human genome copy = 2.85 x 10^5 copy of human genome = 2.85 x 225 x 10^5 18S rDNA copy number = 6.41 x 10^7 18S rDNA copy number. Average genomic DNA extracted from fresh human tissue using Protease K digestion and phenol chloroform precipitation method is 1.98 µg per mg human tissue (based

on 30 human breast capsule tissue). One mg human tissue would have 1.98 x 6.41 x 10^7 = 1.27 x 10^8 copy number of 18S rDNA. 16S rDNA copy number per mg tissue = 16s rDNA copy number (copies/µl) x 1.27 x 10^8 / 18S rDNA copy number (copies/µl). Total bacterial number per mg tissue = 16S rDNA copy number per mg tissue / 5.

5.9.4 PCR amplification and Amplicon sequencing

DNA sequencing will be carried out by a commercial laboratory (Australian Centre for Ecogenomics, Brisbane, Australia) targeting the V3-V4 region of the 16S rDNA using eubacterial universal primers 515F and 806R [315]. Preparation of the 16S rDNA library will be performed using the workflow outlined by the manufacturer (Iluumina Inc. Part # 15044223 Rev. B).

In the 1st stage, PCR products are amplified according to the specified workflow with an alteration in polymerase used to substitute Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, Massachusetts, United States). Resulting PCR amplicons are purified using Agencourt AMPure XP beads (Beckman Coulter). Purified DNA is indexed with unique 8bp barcodes using the Illumina Nextera XT 384 sample Index Kit A-D (Illumina FC-131-1002) in standard PCR conditions with Q5 Hot Start High-Fidelity 2X Master Mix. Indexed amplicons will be pooled together in equimolar concentrations and sequenced on the Illumina MiSeq platform using paired end sequencing with V3 300bp chemistry.

5.9.5 Fluorescence in situ Hybridization (FISH)

Biopsy material embedded in optimal cutting temperature (OTC) embedding matrix (Fisher Scientific, Walthamm Massachusetts, United States), frozen at –80^{o}C, will be cryo-sectioned

to a thickness of 6 μm and mounted on SuperFrost Plus slides (Menzel-Glaser, Lomb Scientific, Australia). Different types of probes will be utilized for in situ hybridization as previously described by Thurnheer [335]. For PNA-FISH, probes and kits will be sourced commercially (AdvanDx, Inc., Woburn, MA) using previously described methods[13].

Briefly, either species-specific probes or universal bacterial probes will be utilised. A hybridization solution is added drop wise to each tissue section and hybridized at 55°C for 90 min. Slides are washed for 30 min at 55°C in wash solution. Once dry, a coverslip is mounted using a single drop of mounting medium. Slides will be examined using CLSM (Zeiss Axio Imager Microscope and/or ZEISS LSM 880, Carl Zeiss Ltd., Herefordshire, UK). Images are processed using ZEISS ZEN Imaging Software (black edition) and Imaris v 8.4, ImarisXT, Bitplane.

5.10 Duration of patient participation

Participants in cohort 1 will be identified when they attend for their appointment. A tissue biopsy will be taken during this time; therefore, an additional appointment will not be required solely for obtaining a tissue biopsy in this cohort. As such participant duration will only be required for the removal of the tissue biopsy over one, 30-minute appointment.

Participants in cohort 2 will be required to attend seven consecutive daily appointments for the application of the antimicrobial wound irrigation solution and antimicrobial hydrogel. Appointments will last for 20 minutes approximately. In people with chronic wound infection the average number of dressing changes by a health professional over a weekly period can range from two to four visits / dressing changes over the week. As such the schedule of consecutive visits does not alter greatly from routine care.

Participants in cohort 3 will be required to attend two, 30 minute appointments over the 7 days. Application of the antimicrobial ointment will be applied at day 1 and at day 4. This is in keeping with a normal dressing regime and does not constitute any additional visits to standard care.

All participants may continue to be followed in the high-risk foot service as part of their routine care for their wound after the completion of the study.

5.11 Source Data

Standardised Clinical Report Forms (CRFs) will be used for data collection.

CRFs will be labelled with a unique identifier, de-identifying the data. However as per routine clinical practice, master lists of patients and their unique identifiers will be held. It is possible that study data will be re-identifiable but all master lists will be held securely in locked filing cabinets with access only given to clinical trials staff.

CRFs will be entered into a secured (by password and 128-bit industry standard encryption) centralised database. Access to the database will only be provided to clinical trials staff.

No identifying data will be published.

Source data and analyses will be held in secure and locked storage for the required period according to relevant legislation.

6. Withdrawal of patients

6.1 Patient withdrawal criteria

Patients may be withdrawn from the study if:

- The chronic wound deteriorates prior to the completion of the last tissue biopsy and a change in treatment regime is clinically indicated such as surgical intervention, change in oral or intravenous antimicrobial, application of topical antimicrobial is applied.

or:

- Withdrawal of consent

6.3.2 Patient drop out

Recruitment will continue to make up for patients who withdraw or who are lost to follow up.

7. Treatment of patients

7.1 Additional appointments

Where the consecutive days of treatment fall on a weekend, it is planned to use community nursing to change the participants wound dressing and apply the antimicrobial wound products. It is standard practice for the high-risk foot clinic to utilise community nursing for weekend home visits to change the dressings of patients. In this scenario, the community nurse will be provided with an instruction procedure on how to apply the products and the dressing regime.

In some instances, it will not be possible to arrange community nursing over the weekends. If this occurs the podiatrist will teach the participant how to apply the products and provide them with a product application leaflet.

7.2 Monitoring adherence

The advantage of following participants in cohort two for seven consecutive days is so that the application of the antimicrobial wound products can be confirmed.

8. Statistics

8.1 Methods and significance levels

For cohort one, descriptive statistics will be calculated appropriate to the data. Comparisons with results in the published literature will be made as appropriate.

For cohorts two and three an analysis of biofilm structure will be utilized to determine effect using the parameters; species composition and the total number of bacteria. A student's t-test will be used to determine the difference in biofilm structure before and after treatment.

All analyses will be done using the Statistical Package for Social Sciences (SPSS Inc., Chicago, Illinois, USA).

8.2 Sample size

Phase I of this study a sample size of 30 patients is deemed adequate to draw conclusions. For phase II, an additional 30 patients (15 per cohort) will be recruited. We estimate that n=30 patients will accommodate a 15% drop out rate while retaining sufficient statistical power to estimate the effect size and variance of the effects of each wound treatment.

9. Study timeline

JAN – MAR 2015	APRL – JUNE 2015	JUL – SEPT 2015	OCT – DEC 2015
Cohort 1 (patients 1-8) * **Begin recruitment** ***** Screening for inclusion * Tissue biopsy taken and split into 3. * Freeze tissue sample for PCR * Send tissue sample immediately for FISH / CLSM / Lab culture.	**Cohort 1** (patients 9-15) * Screening for inclusion * Tissue biopsy taken and split into 3. * Freeze tissue sample for PCR * Send tissue sample immediately for FISH / CLSM / Lab culture.	**Cohort 1** (patients 16-22) * Screening for inclusion * Tissue biopsy taken and split into 3. * Freeze tissue sample for PCR * Send tissue sample immediately for FISH / CLSM / Lab culture.	**Cohort 1** (patients 22-30) * Screening for inclusion * Tissue biopsy taken and split into 3. * Freeze tissue sample for PCR * Send tissue sample immediately for FISH / CLSM / Lab culture. * process frozen tissue samples for PCR analysis in batches of 5.
JAN – MAR 2016	**APRL – JUNE 2016**	**JUL – SEPT 2016**	**OCT – DEC 2016**
Cohort 2 and 3 (patients 31-38) *__Begin recruitment__ ***** Screening for inclusion ***** Scheduled daily visits commence for 5 days. * Tissue biopsy day 1 and day 5. Sample split into 3. * Send tissue samples immediately for FISH / CLSM / Lab culture * Freeze tissue sample for PCR.	**Cohort 2 and 3** (patients 39-46) * Screening for inclusion * Scheduled daily visits commence for 5 days. * Tissue biopsy day 1 and day 5. Sample split into 3 * Freeze tissue sample for PCR until study end. * Send tissue sample immediately for FISH / CLSM / Lab culture.	**Cohort 2 and 3** (patients 47-54) * Screening for inclusion * Scheduled daily visits commence for 5 days. * Tissue biopsy day 1 and day 5. Sample split into 3 * Freeze tissue sample for PCR until study end. * Send tissue sample immediately for FISH / CLSM / Lab culture.	**Cohort 2 and 3** (patients 55-60) * Screening for inclusion * Scheduled daily visits commence for 5 days. * Tissue biopsy day 1 and day 5. Sample split into 3 * Freeze tissue sample for PCR until study end. * Send tissue sample immediately for FISH / CLSM / Lab culture. *Study end process all sample

10. Ethical considerations

The study will conform to the Australian Code for Responsible Conduct of Research (http://www.nhmrc.gov.au/guidelines/publications/r39) and the National Statement on Ethical Conduct in Human Research 2007 - Updated 2009.

(http://www.nhmrc.gov.au/guidelines/publications/e72).

Study related monitoring, audits, HREC review and regulatory inspections will be permitted at all sites with direct access to source data as required. Any conflicts of interest from any investigating staff will be declared before commencement of the study. All patients entering the study will provide informed consent. Patient confidentiality will be respected. No data identifying individual patients will be published.

11. Insurance and financing

11.1 investigator-initiated study

South Western Sydney LHD will assume the risk for the study conducted on its premises as this is an investigator-initiated study. Internal finances will be utilised to fund the study (Allied Health SP&T 588298).

11.2 Study costs

The bulk of study costs are related to the advanced molecular and microscopy techniques. The costs have been broken down into the two study phases. Phase I represents the analysis for a student PhD and as such labour costs are excluded. This phase will be self-funded from a Liverpool Hospital internal research trust fund. Phase II is an analysis of a woundcare product and the calculations of costs for this phase will include labour costs.

Phase I

FISH analysis will be undertaken at Macquarie University and will cost $100 for each sample. At 30 samples the cost for FISH will be $3000. Pyrosequencing for bacterial identification will be undertaken at the Ingham Institute of Applied Medical Research and will cost $150 per sample. This equates to 30 samples at a cost of $4500. Access fee for the CLSM and sample preparation reagents, stubs, gold coating comes to $50 per sample. At 30 samples the cost for

CLSM will be $1500. Laboratory based culture and quantitative analysis will be undertaken at SWAPS and will cost $50 per sample at 30 samples - $1,500.

The total costs to cover cohort 1 will be $10,500

Phase II

CLSM, Fish analysis and pyrosequencing will be undertaken at Macquarie University and will cost $15,000 exc GST for 15 participants for each cohort, total cost $30,000 exc GST. This is calculated at 15 participants requiring analysis of two tissue samples, SEM $200 per sample, FISH $150 per sample, Pyrosequencing $150 per sample.

12. Publication Policy

It is intended to publish the data in International Medical Journals in addition to presenting at national and International conferences.

13. Reimbursement of participants

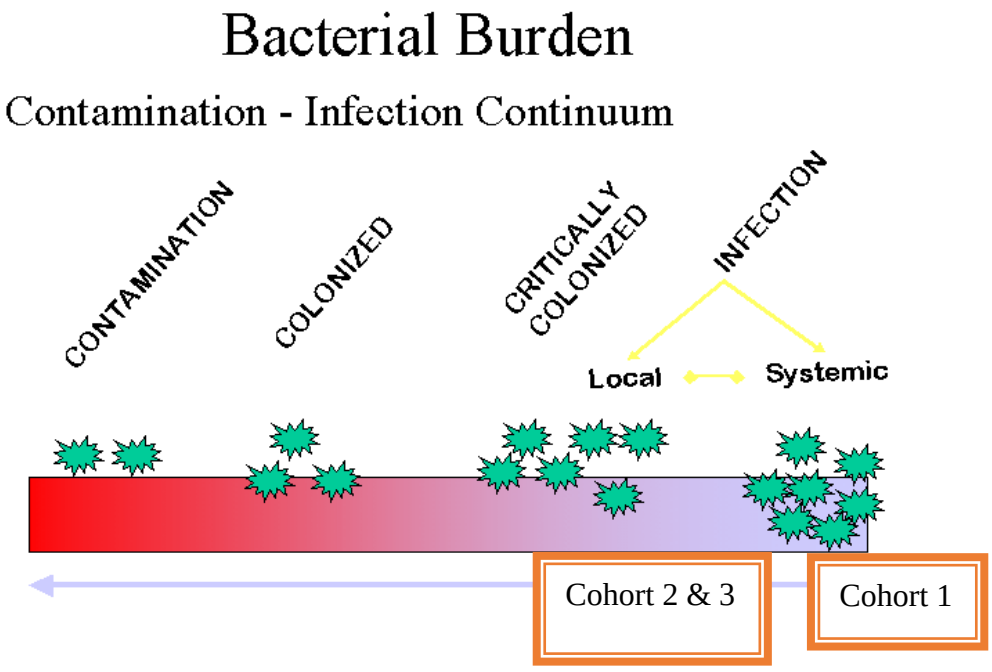
We do not plan to reimburse participants for their time in this study, as the treatment forms part of routine practice.

14. Supplementary material

IDSA Guidelines for diabetic foot infection

Clinical Manifestation of Infection	PEDIS Grade	IDSA Infection Severity
No symptoms or signs of infection	1	Uninfected
Infection present, as defined by the presence of at least 2 of the following items: • Local swelling or induration • Erythema • Local tenderness or pain • Local warmth • Purulent discharge (thick, opaque to white or sanguineous secretion)		
Local infection involving only the skin and the subcutaneous tissue (without involvement of deeper tissues and without systemic signs as described below). If erythema, must be >0.5 cm to ≤2 cm around the ulcer. Exclude other causes of an inflammatory response of the skin (eg, trauma, gout, acute Charcot neuro-osteoarthropathy, fracture, thrombosis, venous stasis).	2	Mild
Local infection (as described above) with erythema > 2 cm, or involving structures deeper than skin and subcutaneous tissues (eg, abscess, osteomyelitis, septic arthritis, fasciitis), **and** No systemic inflammatory response signs (as described below)	3	Moderate
Local infection (as described above) with the signs of SIRS, as manifested by ≥2 of the following: • Temperature >38°C or <36°C • Heart rate >90 beats/min • Respiratory rate >20 breaths/min or $PaCO_2$ <32 mm Hg • White blood cell count >12 000 or <4000 cells/μL or ≥10% immature (band) forms	4	Severe[a]

Bacterial Bioburden, from contamination to infection



Clinical signs and symptoms checklist for infection.

Clinical Signs and Symptoms Checklist (CSSC)	√ if present
Increasing pain in the ulcer area: subject's subjective report of perceived increases in level of peri-ulcer pain since the ulcer developed. Ask subject to select the most appropriate statement for current level of ulcer pain from the following choices. 1) I am not able to detect pain in ulcer area, 2) I am having less ulcer pain now than I have had in the past, 3) the intensity ulcer pain has remained the same since the ulcer developed, or 4) I have more ulcer pain now than I have had in the past. Circle the number corresponding to the most appropriate statement. If number 4 is selected, place a check in the box to the right; mark N/A if subject not able to respond to question.	
Erythema: presence of bright or dark red skin or darkening of normal ethnic skin color immediately adjacent to the ulcer opening.	
Edema: presence of shiny, taut skin or pitting impressions in the skin adjacent to the ulcer but within 4 cm from the ulcer margin. Assess pitting edema by firmly pressing the skin within 4 cm of ulcer margin with a finger, release and waiting 5 seconds to observe indentation.	
Heat: detectable increase in skin temperature of the skin adjacent to the ulcer but within 4 cm of the ulcer margin as compared to the skin 10 cm proximal to the wound. Assess differences in skin temperature using the back of the examiner's hand or the wrist.	
Purulent exudate: presence of tan, creamy, yellow, or green thick fluid on a dry gauze dressing removed from the ulcer one hour after placement. The wound was cleansed prior to placing the gauze dressing in the ulcer.	
Sanguinous exudate: presence of bloody fluid on a dry gauze dressing removed from the ulcer one hour after placement.	
Serous exudate: presence of thin, watery fluid on a dry gauze dressing removed from the ulcer one hour after placement.	
Delayed healing of the ulcer: subject or caregivers report of no change or an increase in the volume or surface area of the ulcer over the past 4 weeks. Ask subject or caregiver if the ulcer has filled with tissue or is smaller around than it was 4 weeks from today. If they report it has not, place check in box to the right.	
Discoloration of granulation tissue: granulation tissue that is pale, dusky or dull in color	
Friable granulation tissue: bleeding of granulation tissue when gently manipulated with a sterile cotton-tipped applicator.	
Pocketing at base of wound: presence of smooth, nongranulating pockets of ulcer tissue surrounded by beefy red granulation tissue.	
Foul odor: putrid or distinctively unpleasant smell as assessed by the examiner.	
Wound breakdown: small open areas in newly formed epithelial tissue not caused by re-injury or trauma.	

Clinical indictors to determine the presence of wound biofilm.

Table 1. Clinical indicators of biofilm in chronic wounds and supporting evidence.

Excessive moisture / exudate	Evidence that excessive moisture encourages biofilm development[12]
Poor-quality granulation tissue (e.g. friable, hypergranulation)	High bioburden may present as friable granulation tissue[13]
Signs and symptoms of local infection	Secondary signs of infection are more typical of biofilm infection[14]
Antibiotic failure or recurring infection following antibiotic cessation	Antibiotic failure is the hallmark of biofilm infection. The use of antibiotics is still controversial regarding biofilm management; it has been suggested that – without the use of concurrent strategies for biofilm management – efficacy may be as low as 25%–30%[15,16]
Negative wound culture	Routine cultures will only pick up the free-floating (i.e. planktonic) bacteria, not those within a biofilm[17,18]
Nonhealing in spite of optimal wound management and host support	Biofilm defences include resistance to: ultraviolet light, biocides, antibiotics and host defences. Biofilm can quickly reconstitute but strategically does not kill its host[19]
Infection lasting >30 days	Infections of ≤30 days' duration may also contain biofilm, planktonic infection would not persist >30 days[15]
Responds to corticosteroids and TNF- alpha inhibitors	Inflammation is a by-product of biofilm, thus a good response to these treatments suggests presence of biofilm. Decreasing inflammation removes the primary source of nutrition[15]
Gelatinous material easily removed from the wound surface	Clinicians and researchers are trying to determine if the by-product of biofilm formation can be clinically seen. Case studies demonstrate differences in wound material that can be easily removed but quickly reform, either on the wound or under a dressing. Some authors believe that slough equals biofilm, but this has not been conclusively proven. A build-up of self-secreting polymers and host components is suggestive of biofilm[20,21]
Surface substance reform quickly	Research suggests that biofilm can reform within 24–72 hours[22]

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11 December 2014

Mr Matthew Malone
Podiatric Medicine
Liverpool Hospital

Dear Mr Malone,

*****THIS LETTER CONSTITUTES ETHICAL APPROVAL ONLY. THIS RESEARCH PROJECT MUST NOT COMMENCE AT A SITE UNTIL SEPARATE AUTHORISATION FROM THE CHIEF EXECUTIVE OR DELEGATE OF THAT SITE HAS BEEN OBTAINED. ******

Project Title: **The Microbiome of chronic wounds**
HREC Reference: **HREC/14/LPOOL/487**
SSA Reference: **SSA/14/LPOOL/489**
Local Project Number: **14/282**

Thank you for your response received 24 November 2014 to our request for further information dated 17 November 2014. This Human Research Ethics Committee is constituted and operates in accordance with the National Health and Medical Research Council's *National Statement on Ethical Conduct in Research Involving Humans* and the *CPMP/ICH Note for Guidance on Good Clinical Practice*.

I am pleased to advise that the Committee has granted ethical approval of the above project.

The following documentation has been reviewed and approved:

Document	Version	Date
National Ethics Application Form	AU/1/BEFA114	07/10/2014
Protocol	1.0	12/08/2014
MASTER Participant Information Sheet (Cohort 1)	2.0	24/11/2014
MASTER Participant Information Sheet (Cohort 2)	2.0	24/11/2014
MASTER Consent Form	2.0	24/11/2014
MASTER GP Letter Cohort 1	1.0	26/08/2014
MASTER GP Letter Cohort 2	1.0	26/08/2014
MASTER Nurse Letter Cohort 2	1.0	26/08/2014
MASTER Initial Screen	1.0	01/09/2014
MASTER Data Collection Sheet	1.0	01/09/2014
Product Information – Iodosorb Cadexomer Iodine Medicated Ointment	Nil	Undated

Please ensure for all future documents submitted for review include a document version number, document date and page numbering.

Monitoring Requirements:
(National Statement Chapters 2.1 and 5.5)

- The Committee has classified this project as:

Low Risk

- Monitoring required for this study will be:
  - Submission of Annual Progress Reports with the first report due **11 December 2015 and annually thereafter for the duration of the approval period**

Approval has been granted for the following site(s):

- Liverpool Hospital

Please note the following conditions of approval:

- **The Committee has granted approval for phase I of the study, but require an amendment to be submitted prior to approval of phase II, once funds have been secured.**
- **Insert Local Project Number 14/282 at the end of the complaints paragraph**
  Please note the Office does not require a copy of the updated Participant Information Sheet and Consent Form. Please amend these before issuing to Participants

1. The Principal Investigator will immediately report anything which might warrant review of ethical approval of the project in the specified format, including:
   - any serious or unexpected adverse events; and
   - unforeseen events that might affect continued ethical acceptability of the project.

2. The Principal Investigator will report proposed changes to the research protocol, conduct of the research, or length of HREC approval to the HREC in the specified format, for review. For multi-centre studies, the Chief Investigator should submit to the Lead HREC and then send the amendment approval letter to the investigators at each sites so that they can notify their Research Governance Officer.

3. The Principal Investigator will inform the HREC, giving reasons, if the project is discontinued before the expected date of completion.

4. The Principal Investigator will provide an annual report to the HREC and at completion of the study in the specified format.

5. The Principal Investigator must reassure participants about confidentiality of the data.

6. Proposed changes to the personnel involved in the study are submitted to the HREC accompanied by a CV where applicable.

HREC approval is valid for (5) years. If the study is ongoing at the conclusion of the five year approval period, a full resubmission may be required. Ethics approval will continue during the re-approval process.

Optional: It is the responsibility of the sponsor or the principal (or co-ordinating) investigator to register this study on a publicly available online registry (eg Australian New Zealand Clinical Trials Registry www.anzctr.org.au)

The South Western Sydney Local Health District Human Research Ethics Committee has been accredited by the NSW Ministry of Health to provide single ethical and scientific review of research proposals conducted within the NSW public health system and Victorian and Queensland Public Health Organisations participating in the Mutual Acceptance Scheme.

You are reminded that this letter constitutes ethical approval only. This research project must not commence at a site until separate authorisation from the Chief

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Research Paper

Next Generation DNA Sequencing of Tissues from Infected Diabetic Foot Ulcers

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ABSTRACT

We used next generation DNA sequencing to profile the microbiome of infected Diabetic Foot Ulcers (DFUs). The microbiota was correlated to clinical parameters and treatment outcomes to determine if directed antimicrobial therapy based on conventional microbiological cultures are relevant based on genomic analysis. Patients ≥ 18 years presenting with a new Diabetic Foot Infection (DFI) who had not received topical or oral antimicrobials in the two weeks prior to presentation, were eligible for enrolment. Tissue punch biopsies were obtained from infected DFUs for analysis. Demographics, clinical and laboratory data were collected and correlated against microbiota data. Thirty-nine patients with infected DFUs were recruited over twelve-months. Shorter duration DFUs (<six weeks) all had one dominant bacterial species ($n = 5$ of 5, 100%, $p < 0.001$), *Staphylococcus aureus* in three cases and *Streptococcus agalactiae* in two. Longer duration DFUs (≥six weeks) were diversely polymicrobial ($p < 0.01$) with an average of 63 (range 19–125) bacterial species. Severe DFIs had complex microbiomes and were distinctly dissimilar to less severe infections ($p = 0.02$), characterised by the presence of low frequency microorganisms. Nineteen patients (49%) during the study period experienced antimicrobial treatment failure, but no overall differences existed in the microbiome of patients who failed therapy and those who experienced treatment success ($p = 0.2$). Our results confirm that short DFUs have a simpler microbiome consisting of pyogenic cocci but chronic DFUs have a highly polymicrobial microbiome. The duration of a DFU may be useful as a guide to directing antimicrobial therapy.

1. Introduction

In a person with diabetes a "triad" of factors that include peripheral neuropathy, peripheral vascular disease and trauma (Lipsky et al., 2012), place the foot at risk of developing a wound. Infections of the feet in people with diabetes are the primary pathway to lower extremity amputation (Lavery et al., 2003). The management of diabetes foot infection (DFI) is underpinned by the requirement to identify the pathogen/s of infection and thus direct antimicrobial therapy. Laboratory based methods that are culture-dependent have been utilised to identify planktonic microorganisms that are potential pathogens of infection, in addition to examining their density through qualitative and quantitative measures. This has shown acute ulcers are usually colonised by *Staphylococcus aureus* and/or *Streptococcus agalactiae* (Group B *Streptococcus*), and chronic ulcers have a more diverse microbiome, with anaerobic organisms and *Pseudomonas aeruginosa* becoming more important (Lipsky et al., 2016a,b). Culture-dependent techniques select for species that flourish under the typical conditions of the diagnostic microbiology laboratory, and this may not necessarily reflect the most abundant or clinically important microorganisms in DFIs especially anaerobes and species not detected under standard clinical microbiology laboratory protocols (Grice et al., 2008).

Molecular DNA based techniques that are culture-independent have identified the limitations of traditional cultivation based methods when examining the microbiome of wounds. Using amplification and sequence analysis of 16s rDNA, a highly-conserved gene present in all prokaryotes (bacteria) but not eukaryotes (humans), has revealed a vastly more complex array of bacterial communities in non-infected chronic wounds (Dowd et al., 2008a,b; Gardner et al., 2013; Rhoads et al., 2012a,b; Smith et al., 2016). No data exists for acutely infected DFUs using this methodology.

We explored the microbiome of infected DFUs using next generation DNA sequencing. Data is presented on the microbial diversity,

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community structure, bacterial load and presence of likely pathogens from diabetic foot infections. Molecular findings are correlated against clinical factors and treatment outcomes.

2. Materials and Methods

2.1. Patients, Samples and Ethics

Individuals presenting to a tertiary referral hospital (Liverpool Hospital High Risk Foot Service and Liverpool Hospital Emergency Department) with a newly infected diabetic foot ulcer occurring below the malleolus (Lipsky et al., 2012) were recruited consecutively over a twelve-month study period between January 2015 and December 2015. A 3 mm (width) × 10 mm (depth) tissue punch biopsy was obtained from the edge of each DFU after debriding and cleansing the wound with NaCl 0.9%. Patients who had received any systemic or topical antimicrobial therapy two weeks prior to enrolment were excluded. Ethics approval for this study was granted by the South West Sydney Local Health District Research and Ethics Committee (HREC/14/LPOOL/487, SSA/14/LPOOL/489). The study methodology was designed in guidance by STROME-ID and our molecular surveillance data are reported in keeping with this (Field et al., 2014).

2.2. Patient Demographic, Laboratory and Clinical Data

Patient demographics, laboratory and clinical data were collected through patient charts and the electronic medical records for correlation against microbiome data. Clinical data and wound metrics of interest that were collected included; present or absent foot pulses, foot doppler waveforms, toe brachial indices (TBI) and completion of the modified neuropathic disability score (Abbott et al., 2002). DFU location, duration of DFU prior to presentation, size (length x width in mm), depth (mm) and tissue type (granulation, slough, necrosis). Laboratory data included; full blood count, inflammatory markers (White cell count [WCC], Erythrocyte sedimentation rate [ESR], C-reactive protein [CRP]), glycosylated haemoglobin (HbA1c) and estimated glomerular filtration rate (eGFR). All newly infected DFUs were diagnosed clinically, and their severity graded using the Infectious Disease Society of America Guidelines for DFI (Lipsky et al., 2012). Acute infections were defined based on new presenting symptoms (classic signs of infection) being present and untreated of less than fourteen days duration.

DFUs were classified based on their duration, with shorter duration DFUs (Acute) being less than six weeks and longer duration DFUs (Chronic) defined as those greater than six weeks. Treatment failure during the study period were defined as no resolution of infective symptoms over an appropriate treatment period (>28 days) despite directed anti-infective treatment (Lipsky et al., 2012), a requirement to replace oral antimicrobial therapy with parenteral delivery due to deterioration of infective symptoms, or the need for surgical intervention.

2.3. Specimen Collection, Sampling Processing and the Work Flow for Undertaking Molecular and Culture Dependent Approaches

Specimen collection, storage and the work flows for performing DNA extraction, next generation DNA sequencing, sequence analysis and quality control of DNA reads and qPCR to determine the microbial load, were performed as previously described by our group (Malone et al., 2017) and can be found in supplementary appendix (S1). Culture-dependent bacteriological enumeration and identification from tissue cultures was performed by a hospital pathology service (Sydney South West Pathology Service) using methods previously described (Oates et al., 2014).

2.4. Statistics

CLC genomics workbench version 8.5.1 in combination with the microbial genome-finishing module (CLC bio, Qiagen Aarhus, Denmark) were used to analyse DNA sequence data. Operational taxonomic units (OTU) clustering were based on previously reported wound microbiome analysis (Gardner et al., 2013). OTUs were defined as molecular proxies for describing organisms based on their phylogenetic relationships to other organisms. Associations between microbiome community structure and membership were compared using permutational multivariate analysis of variance (PERMANOVA) in combination with principal coordinate analysis PCoA Bray-curtis dissimilarity matrix. Patient demographics, laboratory and clinical data were examined using Chi-square and Spearman correlation coefficients. Kappa coefficients were used to determine the level of agreement between culture-dependent approaches and DNA sequencing. Independent predictors of treatment failure were explored using general linear model (GLM). Mann Whitney *U* test for non-parametric data were undertaken when analysing the subgroups of neuropathic or neuroischemic lesions. Analysis was performed using Statistical Package for Social Sciences (SPSS) Version 23, SPSS Inc., Chicago, Illinois, USA. For all comparisons and modelling, the level of significance was set at $p < 0.05$. Molecular data analysed through Bray-curtis and PERMNOVA incorporated a Bonferroni correction. Data are given as mean, median and standard deviation (±).

3. Results

39 patients (39 tissue specimens) with newly infected DFUs were recruited over the 12-month study period. Broad demographic, clinical and laboratory data are shown in Table 1. Next generation DNA sequencing generated 1,028,895 sequences, which were clustered and

Table 1
Patient demographics, clinical and laboratory data for 39 patients presenting with diabetic foot infection.

Characteristics	n = patients (%) (±SD)
Demographics	
Mean age	57.4 years (±11.5)
Male/female	28 (71%)/11 (29%)
Type of diabetes: type 1/type2	4 (10%)/35 (90%)
Duration of diabetes	12.8 years (±6.5)
Chronic kidney disease stage 5	16 (27%)
Duration of ulcer prior to presentation	15.7 weeks (±13.7)
Co-morbidities	
Loss of protective sensation	39 (100%)
Peripheral arterial disease	15 (38.5%)
Toe brachial index	0.5 (±0.1)
Laboratory data	
Glycosylated haemoglobin (HbA1c) (%)	8.5 (±2.5)
Erythrocyte sedimentation rate (mmol/L)	54.3 (±33)
C-reactive protein (mg/l)	28.1 (±25)
White cell count	9.2 (±2.4)
Infection grading and classification (IDSA)	
Mild	5 (13%)
Moderate	25 (64%)
Severe	9 (23%)
Systemic antimicrobial/route of delivery	
Cephalexin/oral	6 (15%)
Amoxycillin + clavulanic acid/oral	13 (33.5%)
Flucoxacillin/oral	3 (8%)
Clindamycin/oral	1 (2.5%)
Ciprofloxacin/oral	1 (2.5%)
Rifampin + fusidic acid/oral	2 (5%)
Sulfamethoxazole + trimethoprim/oral	1 (2.5%)
Combination therapy/oral	3 (8%)
Piperacillin + tazobactam/intravenous	6 (15%)
Cephazolin/intravenous	3 (8%)

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aligned at 97% similarity to reveal 1139 unique OTUs. A total of seven major phyla were identified including Firmicutes (48%), Proteobacteria (26%), Actinobacteria (12%), Bacteroidetes (8%), Fusobacteria (2%) and Cyanobacteria (1%). The clustering of OTUs contributing to greater than 10% within each DFU sample at the genera/species-level is noted in Table 2 and those contributing to < 10% are noted in (S2). *Staphylococcus* spp. was the most commonly sequenced microorganism in infected DFUs. This was followed by *Corynebacterium spp.*, *Finegoldia* spp., *Peptoniphilus* spp., *Acinetobacter* spp., *Anaerococcus* spp., and *Streptococcus* spp., We further categorized microorganisms based on their residing niche (environmental, skin, oral and gut) to better define the site of origin of microorganisms that colonize DFUs (S3). Microorganisms commensal to the skin were predominant in half of patients (50.6%) followed by environmental (29.1%), gut (14%) and oral (6.3%) microorganisms.

3.1. Community Structure of DFIs are Heterogeneous

The community structures of DFIs were depicted using rarefaction and Shannon Weaver index plots, which explore the richness, and diversity of individual infected DFUs (Fig. 1). Most DFUs had complex polymicrobial communities with great heterogeneity between patients. Rarefaction identified a mean of 56 OTUs (±31.2, range 4 to 125) per DFU, and Shannon Weaver index identified a mean indices of 2.3 (±0.9, range 0.4–4.1). Descriptive statistics allowed for a more clinically relevant picture to be composed of the overall community structure. We identified three general profiles that sub-divided DFUs based on their community structure (Fig. 2). High frequency taxa mostly comprised of a single microorganism (±3) (i.e. monomicrobial infection), high to low frequency taxa were comprised of between one to five (±2) dominant microorganisms followed by many low frequency taxa (i.e. polymicrobial infection) and low frequency taxa comprised on average of ≥20 (±) minor microorganisms (complex polymicrobial infection).

3.2. The Duration of DFU Prior to Infection Presentation may Present a Major Driver Behind the Microbiome

Five (13%) DFUs at the time of presentation were less than six-weeks in duration, and were composed of high frequency taxa with one predominant microorganism (Fig. 3). These were *S. aureus* in three cases and *S. algalactiae* in two cases. The relative abundance of *Staphylococcus* spp., was far greater in DFUs < six-weeks then DFUs of longer duration where it was present but at significantly lower relative abundances (Fig. 4).

Longer duration DFUs (≥6 weeks) with a new acute infective episode (n = 34, 87%) were the most common presentation. PCoA bray-curtis plots with PERMANOVA identified the community structures between longer and shorter duration DFUs were dissimilar ($p < 0.003$) (Fig. 5 and S4). Furthermore, analysis of variance identifies that longer duration DFUs (>6 weeks) were associated with a greater relative abundance of Proteobacteria ($p < 0.05$), whilst shorter duration DFUs (<6 weeks) were associated with greater relative abundance of Firmicutes ($p < 0.001$). (Fig. 6) Closer examination of OTUs revealed

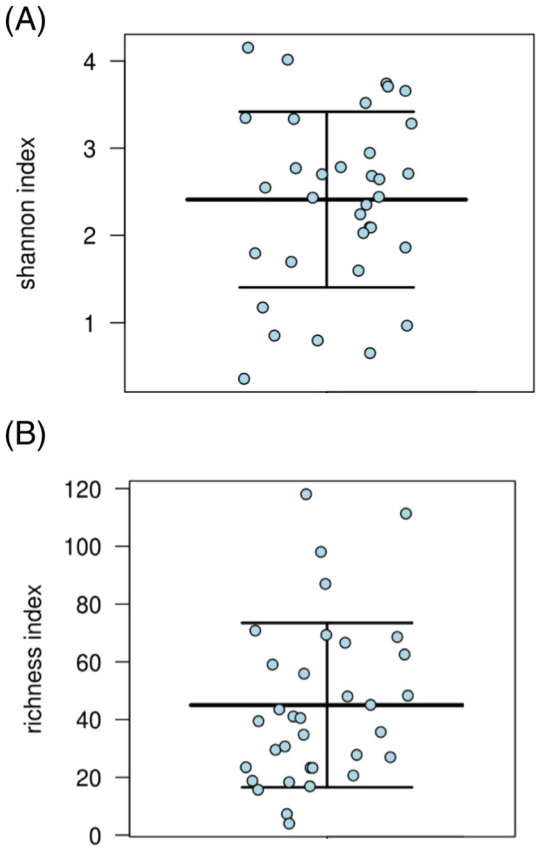

Fig. 1. Community diversity and richness reported for 39 patients with DFI. (A) Community diversity of DFUs presented using the Shannon-Weaver index at maximum read length of 300 (Price et al., 2009). Shannon Weaver Index is a measure of diversity that includes the number of unique microbial taxa and their relative evenness within each sample. Thus, a higher Shannon Weaver Index correlates to a greater diversity. (B) Community richness of DFUs presented using richness index reporting the number of unique OTUs in each wound sample. Data sets were normalised to remove low abundance OTUs contributing too less then 1% within each wound sample.

Table 2
Microorganisms contributing ≥10% in each sample (representing the dominant taxa) (Rhoads et al., 2012a,b).

Genera/species	Samples	Avg abundance %	SD	Min-max avg. abu %	Aerotolernace
Staphylococcus spp.:	15	40.7	30.3	12 to 92	Facultative
Staphylococcus aureus[a]	8	43.1	32.9	12 to 92	Facultative
Unclassified Staphylococcus spp[a]	7	31.8	28.2	12 to 81	Facultative
Staphylococcus pettenkoferi[a]	2	26	3	23 to 29	Facultative
Corneybacterium striatum	8	32	16.6	12 to 59	Facultative
Finegoldia spp.	7	12	2.8	10 to 18	Anaerobe
Peptoniphilus spp.	7	14.5	5.1	10 to 22	Anaerobe
Acinetobacter baumannii	7	30.5	18.7	16 to 69	Facultative
Anaerococcus spp.	6	14.3	5.1	12 to 24	Anaerobe
Streptococcus agalactiae	5	45.2	39	16 to 89	Facultative
Enterobacter spp.	5	19.6	8.1	10 to 28	Facultative
Proteus spp.	4	22.7	4.5	19 to 23	Facultative
Prevotella spp.	4	14.3	4	10 to 18	Anaerobe
Haemophilus spp.	4	21	14	12 to 42	Facultative
Blastocatella fastidiosa	3	24	11	12 to 32	Facultative
Pseudomonas aeruginosa	2	12.5	3.5	10 to 15	Aerobe
Porphymonas spp.	2	11.5	2	10 to 13	Anaerobe

[a] Refers to the species level identification of Staphylococcus genus level data.

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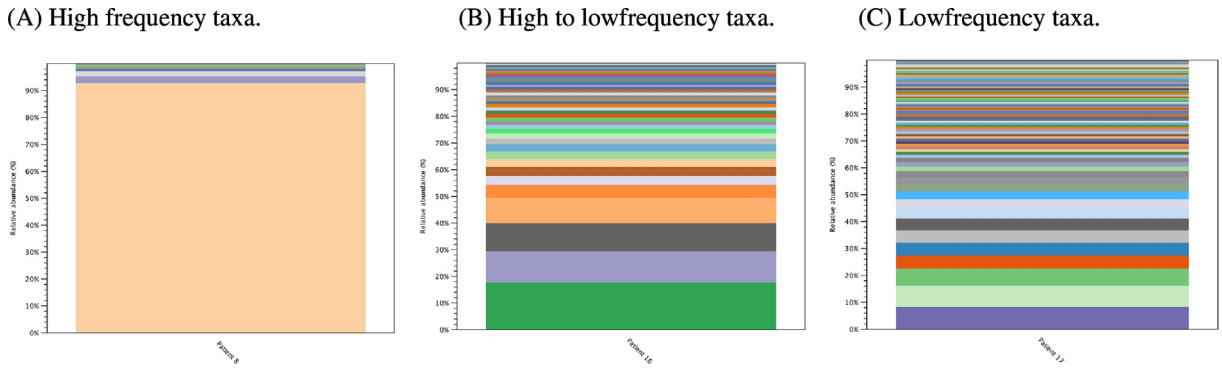

Fig. 2. Bar chart represents relative abundances (%) of taxa profiles for 39 DFUs. Each bar represents individual genera/species. (A) High frequency taxa were observed in ten patients (26%), mostly comprised of a single microorganism (±3) (i.e. monomicrobial infection) contributing to ≥88% (±5.4%) of total abundance. (B) High to low frequency taxa were the most common profile and were observed in 25 patients (64%). Low frequency taxa comprised on average of ≥20 (±) minor microorganisms each contributing <1%–5% abundance and no single microorganism contributing >10% (complex polymicrobial infection). (C) Low frequency taxa were infrequently observed in only four patients (10%) and contained higher relative abundances of environmental microorganisms ($p < 0.01$).

that *Staphylocci* spp., were contributing to the positive correlation detected between Firmicutes and relative abundance and ulcer duration ($p < 0.05$).

Spearman's correlation coefficients further clarified that DFUs of longer duration were polymicrobial, typically having greater number of OTUs and were broader in diversity ($p < 0.01$). This statistical approach further correlated higher frequencies of DFUs containing obligate anaerobes that constituted greater than 30% of the total abundance in DFUs of greater duration ($p < 0.03$) (S5).

3.3. Wound Observations and Clinical Factors Lack Association With the Microbiome

Associations between clinical factors and DFI microbiome were compared using PERMANOVA and spearman rank correlation coefficients. The location, depth and the level of glycosylated haemoglobin (HbA1C ≥ 7%) were not associated to any significant microbiota. The presence of slough or malodour within an infected DFU were independently associated with community structure, but were not inversely correlated to each other ($p = 0.7$). Slough in an infected DFU was associated with higher abundances of obligate anaerobes (slough present and ≥30% anaerobe present = 13 of 39, 33%, $p < 0.01$), as was malodour (malodour DFUs = 15, mean anaerobe abundance 34%, SD25.3 versus no malodour of DFUs, mean anaerobe abundance 15%, SD 18.4).

3.4. Infection Severity of Diabetic Foot Infections are Associated with Altered Community Structures

PERMANOVA identified some disparity between the community structure and infection severity. Mild DFIs were different from both

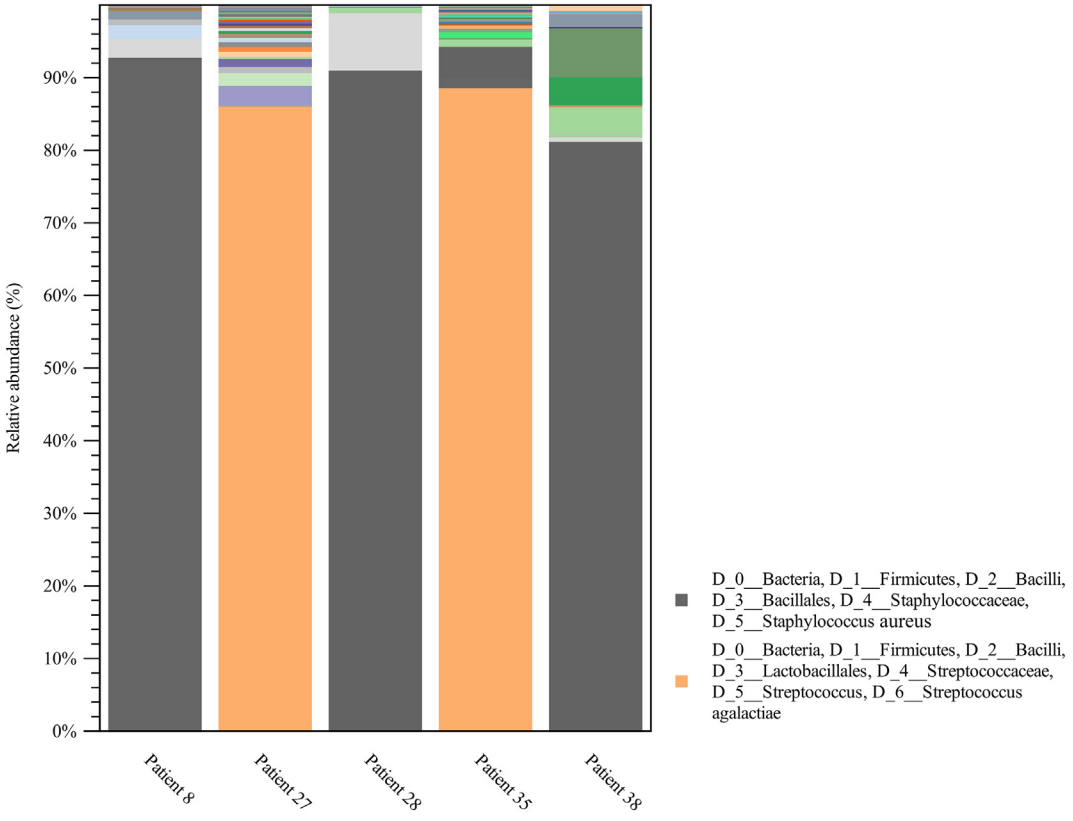

Fig. 3. Bar chart representing relative abundance of taxa in acute diabetic foot ulcers (<6 weeks duration).

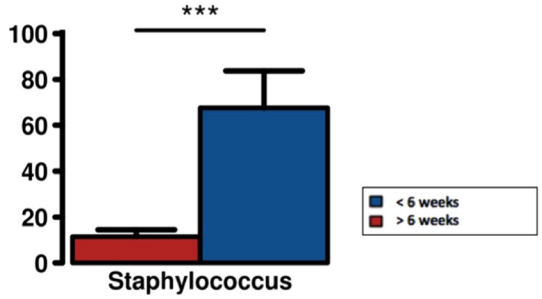

Fig. 4. Analysis of variance between *Staphylocci* spp., relative abundance (%) in DFUs based on duration. In DFUs < six weeks *Staphyloccci* spp., were present as the dominant taxa (high frequency). The average relative abundance of *Staphyloccci* in DFUs > six-weeks is far less and this is because DFUs of longer duration are typically polymicrobial.

moderate infection ($p < 0.01$) and severe infection ($p < 0.001$) (Fig. 7), and were positively correlated to fewer OTUs and were less diverse. In contrast, severe infections often presented exclusively with low frequency taxa profiles ($n = 3$ of 4, $p = 0.02$). Obligate anaerobes and their abundance within each DFU were explored for relationships between infection severities. The abundance of anaerobes was similar across mild DFIs (abundance = 29.5%, ±31) moderate DFIs (abundance = 20.5%, ±22.3) and severe DFIs (abundance = 27.3%, ±21), indicating there exist no differences between patients presenting with more severe infections and a greater abundance of anaerobes ($p = 0.6$).

3.5. Neuropathic and Neuroischaemic Diabetic Foot Ulcers Harbor Similar Microbiomes

Twenty-three patients were classified as having neuropathic DFUs (normal TBI ranges ≥0.9–<1.2 and MNDS ≥ 6) and sixteen patients were classified as having neuroischaemic DFUs (TBI < 0.7 to 0.3, and MNDS ≥ 6). Non-parametric approaches identified no difference in the mean average abundances between neuropathic and neuroischaemic DFUs (S6).

3.6. Culture Dependent Methods Underestimate Anaerobic Microorganisms

Kappa coefficients were used to determine the level of agreement between culture-dependent methods and DNA sequencing. Agreement in the identification of obligate anaerobes was poor between culture and DNA sequencing ($p = 0.4$). Culture underestimated obligate anaerobe presence in 90% of samples (detection of obligate anaerobes by culture = 4 of 39, 10% vs detection of obligate anaerobes by DNA sequencing = 34 of 39, 79%).

3.7. Treatment Parameters and Outcomes

In total, nineteen patients (49%) during the study period experienced treatment failure. Of the thirty-three patients who had DFUs > 6 weeks, fifteen (45%) with moderate to severe IDSA infections experienced treatment failure. In the group of five patients with DFUs < 6 weeks, four patients (80%) with moderate IDSA infections experienced treatment failure. These infections were mono-microbial and were predominated by high frequency taxa of either *Staphylococcus* spp. and *Streptococcus* spp. Correlation coefficients were used to explore if DFUs containing high relative abundances of commonly cited pathogens of infection in DFI (*S. aureus*, *S. agalactiae* and *A. baumannii*) were at greater risk of treatment failure. This revealed the presence of *S. agalactiae* in DFUs (regardless of duration of DFU) were associated with greater treatment failures ($P < 0.007$). PERMANOVA revealed no further differences in the community structures between patients who failed therapy and those who experienced treatment success ($p = 0.2$).

In patient samples where obligate anaerobes were identified as contributing to the overall wound microbiome at levels greater than both 30% and 50%, there were no increased tendency towards failing therapy. The type of ant-infective therapy provided to patients in this study provided adequate anaerobe cover (25 of 39 wounds received antimicrobials with anaerobe cover, 64%) and this may explain the lack of significance between high relative abundance of anaerobes in DFUs and no increased tendency to fail therapy.

Thirteen patients (33.3%) received narrow spectrum antimicrobials with nine (23.1%) of these patients having DFUs > 6 weeks duration. Four of these nine patients (44.4%) experienced treatment failure whilst

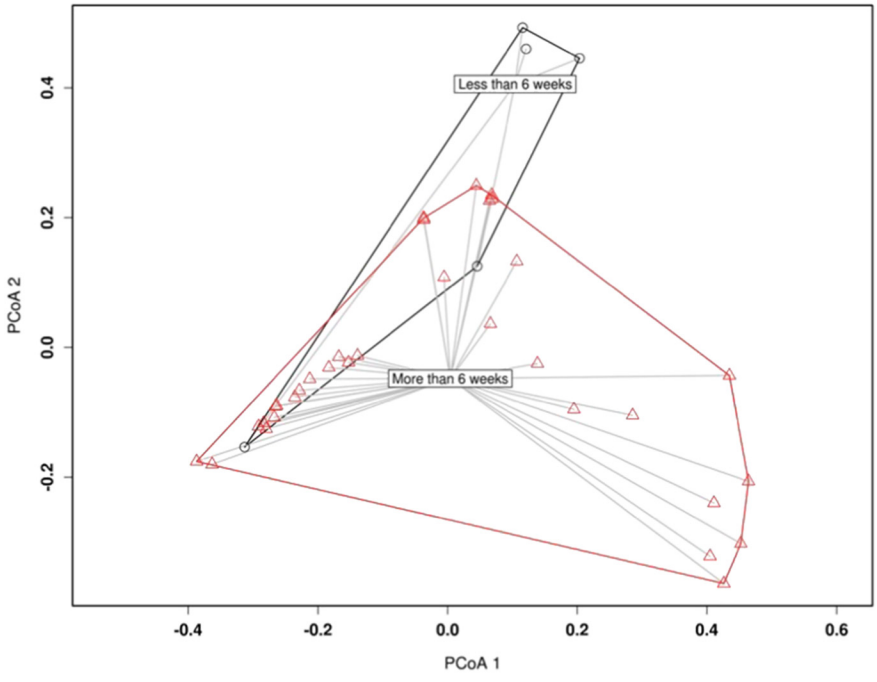

Fig. 5. PCoA bray-curtis plots identify that differences are present in the community structures between longer and shorter duration DFUs.

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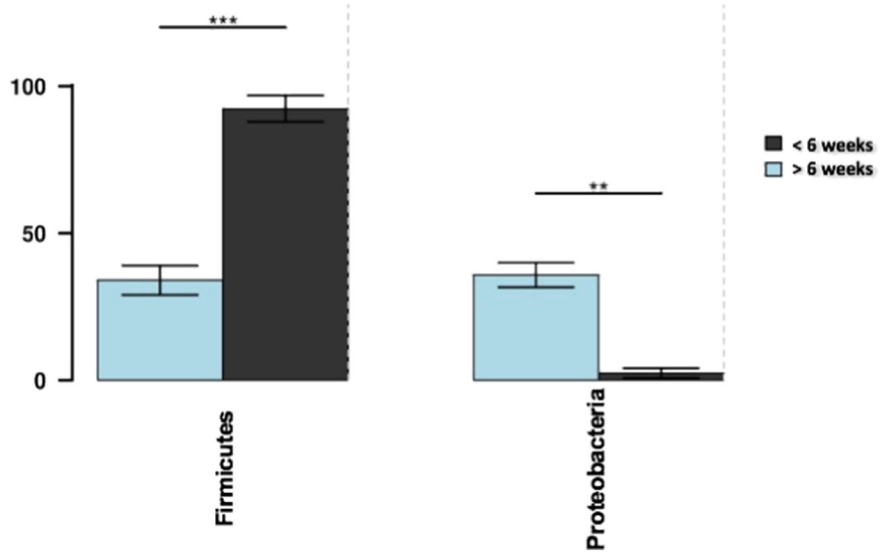

Fig. 6. Analysis of variance identifies that ulcer duration >6 weeks was associated with a greater relative abundance of Proteobacteria ($p < 0.05$), whilst ulcer duration <6weeks was associated with greater relative abundance of Firmicutes ($p < 0.001$). The genera responsible for the high relative abundance of firmicutes in DFUs <6 weeks were *Staphylococcus spp.*, and *Streptococcus spp* predominantly.

receiving narrow spectrum antimicrobials, but the five remaining patients with DFUs > 6 weeks on narrow spectrum antimicrobials experienced treatment success. The number of cases were too low for statistical analysis. Twenty-five patients received broad-spectrum antimicrobials with eleven patients (44%) experiencing treatment failure. There were no correlations between a tendency to fail therapy whether on narrow spectrum or broad-spectrum antimicrobials.

A GLM was performed to identify any predictors of treatment failure independent of the microbiome. These identified patients having a low TBI (<0.7) as being the only predictor of failure regardless of the microbiome ($p = 0.01$). No other clinical factors such as a level of glycosylated haemoglobin greater than ≥7% ($p = 0.72$) or the severity of infection were correlated to treatment failure (Mild, $p = 0.13$, Moderate, $p = 0.65$, Severe, $p = 0.26$).

4. Discussion

In the context of managing diabetes foot infections from an infectious disease viewpoint, current guidelines based on culture-dependent data, are now subject to the scrutiny of molecular DNA based approaches. Furthermore, studies employing amplification and sequence analysis of the 16S rRNA gene to characterize the microorganisms involved in DFI, none to date have sampled participants with overt clinical signs of infection. Instead the available data report on chronic, new or recurrent DFUs that are clinically non-infected (Dowd et al., 2008a,b; Gardner et al., 2013; Price et al., 2009; Smith et al., 2016; Wolcott et al., 2015). Given the increasing utilisation of genomic analysis from both a clinical and research domain, it is essential to understand the microbiota of clinically infective DFUs and if current anti-infective practices can be improved through the translation of complex bioinformatics arising from the DNA analysis of microbial communities. We analysed robust microbiota datasets from Infections of the feet in people with diabetes, and detailed their clinical outcomes, relating this back to current anti-infective practices. We found that the duration of a DFU prior to presenting with a new clinical infection may help clinicians decide on the antimicrobial regimen of choice.

We identify *Staphylococci* spp. as the most commonly sequenced dominant bacteria in approximately one third of DFUs in this study,

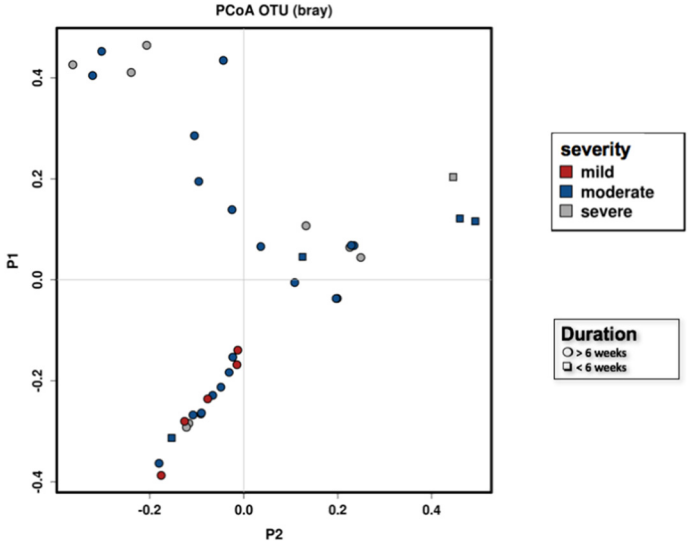

Fig. 7. PCoA Bray-curtis plot demonstrates the community structure difference between infection severities in addition to defining the duration of DFU.

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followed closely by *Corynebacterium* spp. In a recent review by our group on the bacteriology of DFUs from both a molecular and culture based approach (Malone et al., 2016), the predominant pathogen/s of infection for DFI was *S. aureus*. Additionally, *Corynebacterium* spp., *Streptococcus* spp., and obligate anaerobes belonging to *Clostridiales* family XI all identified as major players in this study were similarly identified in studies of chronic non-infected wounds. Based on our molecular data and that of previous molecular and culture based publications, current guidelines that promote the use of antimicrobials targeting Gram-positive aerobic cocci as a first line treatment are appropriate.

Corynebacterium spp. has provided a continuing source of debate regarding its role as a non-pathogenic skin commensal (Citron et al., 2007), or as a pathogen of infection in the presence of an in immunocompromised patient (Dowd et al., 2008a,b; Uçkay et al., 2015). In this study, we seldom identified the presence *Corynebacterium* spp. as a sole pathogen (High frequency taxa), with it almost exclusively occurring in combination with other known pathogens of DFI. This suggests that there may be a role for *Corynebacterium* spp. as part of a polymicrobial infection. Given that many first line antimicrobials of choice for DFI are active against this microorganism, there may not be a requirement to target this sole microorganism unless a mono-microbial culture is identified.

Community structure is essentially the composition of a community, including the number of species in that community, their relative numbers (Richness) and their complexity (Diversity). We identify that the duration of DFU is a major driver behind the microbiome, with longer duration DFUs typically having greater species richness and diversity. This correlated to increased relative abundances of Gram-negative proteobacteria and reduced relative abundances of firmicutes in a pattern previously described by Gardener et al. on neuropathic non-infected DFUs (Gardner et al., 2013). Proteobacteria are commonly identified in wounds (Dowd et al., 2008a; Wolcott et al., 2015) and largely belong to the *Pseudomonadaceae* and *Enterobacteriaceae* families. It is unclear from our data if these microorganisms require special attention, for example *P. aeruginosa* was present as minor taxa in only one quarter of samples (eight DFUs), thus supporting the general consensus (Lipsky et al., 2012) that *P. aeruginosa* is not a typical pathogen of infection in DFI (excluding southern hemisphere locations) (Sivanmaliappan and Sevanan, 2011), and may not require additional tailored therapy should it be identified through cultivation based methods.

Obligate anaerobes were also identified in 90% of DFUs, but great heterogeneity existed between patients with regards to their relative abundances. In most DFUs, obligate anaerobes co-existed in high abundances with aerobic microorganisms, suggesting that obligate anaerobes likely play a role as co-pathogens in DFI. Current microbiology laboratories do not employ enhanced culturing methods to isolate many of the obligate anaerobes identified in this study through DNA sequencing. However, even in the absence of culture-dependent guidance, many commonly utilised antimicrobials for DFI are active against obligate anaerobes.

Furthermore, there are no studies exploring if additional anti-anaerobic therapy improves DFI outcome, and in this study, we find no correlation between the high relative abundance of obligate anaerobes and a greater tendency to fail antimicrobial therapy. The decision to use targeted antimicrobials against obligate anaerobes by clinicians should be administered under the guidance of antimicrobial stewardship (Lipsky et al., 2016a,b). The pattern of antimicrobial therapy prescribing in this study were based on specialist Infectious disease physicians with experience of managing these complex patients, but these results may reflect differently when managed by non-specialist clinicians with limited exposure to these wounds. Further work in this area is required.

The current guidance materials available to clinicians managing DFIs are predominantly based on culture-dependent data, yet this study employing DNA sequencing techniques re-enforces most of this data as being clinically relevant (Lipsky et al., 2012; Lipsky et al., 2016a,b). Pyogenic cocci were predominant in acute DFUs in this study, and thus, supports directed narrow spectrum antimicrobial regimens (with consideration for culture sensitivities looking for *MRSA*). DNA sequencing methods however, highlighted the limitation of conventional bacterial cultures with regards to the microbial diversity and ability to isolate microorganisms not detected under standard clinical microbiology laboratory protocols. Many of these microorganisms were found in chronic wounds that harboured flora similar to the environment, suggesting patients expose their wounds to an array of environments (i.e. barefoot walking, showering with no wound dressing, gardening etc.). Therefore, patient education is vital in order of minimizing exposure of DFUs to environmental contaminants and opportunistic pathogens of infection.

Current guidelines for classifying and managing infected DFUs provide guidance (in conjunction with local policies and patterns of microbial sensitivities for resistance) on the duration and route of delivery of antimicrobials based on infection severity (Lipsky et al., 2012; Lipsky et al., 2016a,b). The use of broad-spectrum antimicrobials delivered parenterally is promoted for severe DFIs, owing to the polymicrobial nature of infection. We confirm severe DFIs are extremely diverse, polymicrobial and complex, and our data supports current clinical practice by parenteral, broad-spectrum antimicrobials is warranted. Exploration from a larger sub-set of patients with severe DFI composing of low frequency taxa profiles, may provide greater insight into managing these challenging infections.

Previous reports have suggested that DFUs complicated by peripheral arterial disease (i.e. ischemic or neuroischemic) likely lead to an altered wound microenvironment and thus microbiota (Gardner et al., 2013). Additionally, the presence of peripheral vascular disease as a comorbid variable in the presence of an infected foot in a person with diabetes has been reported as well known independent predictor of poor outcome (Hinchliffe et al., 2016; Prompers et al., 2008). Sixteen patients in this study had neuroischemic ulcers (TBI < 0.7 to 0.3) with most DFUs presenting with mild to moderate peripheral arterial disease. We ascertained that both neuropathic and neuroischemic (patients with mild to moderate PAD) DFUs harbor similar microbiomes and the requirement to segregate these differing wound aetiologies may not be required for microbiota analysis when using a TBI cut off value of 0.5 as an arbitrary marker.

Nineteen patients during the study period experienced antimicrobial treatment failure, but no differences existed in the microbiome of patients who failed therapy and those who experienced treatment success. Furthermore patients who were treated with either narrow spectrum or broad-spectrum antimicrobials experienced similar failure rates (44.4% versus 44%) and this suggests that other factors are likely at play including the host immune response to infection, patient compliance in adhering to therapy and or peripheral perfusion. A general linear model approach identified that the presence of a TBI < 0.7 was an independent predictor of treatment failure regardless of the microbiota, or antimicrobial, emphasising the difficulties in managing these complex infected wounds.

Whilst our microbiome data identifies DFUs of greater than six-weeks duration presenting with a new clinical infection (includes mild-moderate-severe, with no discrimination) are often polymicrobial, with exception to nearly always targeting aerobic Gram-positive cocci, the requirement to also target provide additional anti-anaerobe therapy requires further research.

Furthermore, whilst DNA sequencing provides an extended view of the microbiome, it is limited in providing information on "which microorganisms" maybe directly contributing to infection. This is increasingly important when analysing our data set where regardless of the spectrum of activity of antimicrobials (i.e. Narrow or broad-spectrum), patients experienced similar outcomes. It may be possible in a highly diverse microbiota, that narrow spectrum antimicrobials targeting pyogenic cocci alone, is enough to reduce the virulence/pathogenicity of infective symptoms without the requirement to use a scatter gun approach to target everything broadly. The use of whole genome

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sequencing may allow us to better understand this question (Malone et al., 2016).

This paper provides a useful insight into the bacterial communities in infected DFUs and reflects on treatment outcomes of anti-infective therapy and if molecular based data would have altered therapeutic regimens. The paper is limited by the sample size and thus recommendations based on molecular data are not possible. A larger cohort of patients would provide greater detail and where possible analysis of small subsets of interest. This paper also identifies the difficulties with obtaining species level data when using the 16s rRNA subunit. Furthermore, what is strikingly apparent from our data is that whilst we provide an extended view of "which microorganism/s" are present, we cannot be definitive on "which microorganism/s" are responsible as contributing as pathogens of infection. The era of "metagenomics" and whole genome sequencing that can analyse genes responsible for virulence or pathogenicity may unveil these answers.

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Conflict of Interests

MM is a paid consultant for Smith and Nephew LTD and has received grant funding from Mundi Pharmaceuticals.

Author Contributions

MM, HD, SJ, IG, KV conceived the study, and MM, HD and IG acted as principal site investigators and were responsible for patient care and acquisition and quality of the data. SJ, KV, KJ, HH were responsible for performing laboratory based activities for DNA methods. MM, SJ and KJ undertook molecular data analysis. All authors were involved in interpretation of the data and participated in writing of the manuscript.

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Appendix A. Supplementary Data

Supplemental information includes a detailed methodology of DNA sequence workflow and statistical analysis. Supplementary data associated with this article can be found in the online version, at doi: http://dx.doi.org/10.1016/j.ebiom.2017.06.026.

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ORIGINAL ARTICLE

Microscopy visualisation confirms multi-species biofilms are ubiquitous in diabetic foot ulcers

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Abstract

Increasing evidence within the literature has identified the presence of biofilms in chronic wounds and proposed that they contribute to delayed wound healing. This research aimed to investigate the presence of biofilm in diabetic foot ulcers (DFUs) using microscopy and molecular approaches and define if these are predominantly mono- or multi-species. Secondary objectives were to correlate wound observations against microscopy results in ascertaining if clinical cues are useful in detecting wound biofilm. DFU tissue specimens were obtained from 65 subjects. Scanning electron microscopy (SEM) and peptide nucleic acid fluorescent in situ hybridisation (PNA-FISH) techniques with confocal laser scanning microscopy (CLSM) were used to visualise biofilm structures. Next-generation DNA sequencing was performed to explore the microbial diversity. Clinical cues that included the presence of slough, excessive exudate, a gel material on the wound bed that reforms quickly following debridement, poor granulation and pyocyanin were correlated to microscopy results. Of the 65 DFU specimens evaluated by microscopy, all were characterised as containing biofilm (100%, $P < 0{\cdot}001$). The presence of both mono-species and multi-species biofilms within the same tissue sections were detected, even when DNA sequencing analysis of DFU specimens revealed diverse polymicrobial communities. No clinical correlations were identified to aid clinicians in identifying wound biofilm. Microscopy visualisation, when combined with molecular approaches, confirms biofilms are ubiquitous in DFUs and form either mono- or multi-species biofilms. Clinical cues to aid clinicians in detecting wound biofilm are not accurate for use in DFUs. A paradigm shift of managing DFUs needs to consider anti-biofilm strategies.

Introduction

Foot ulceration is a physical break in the protective barrier of the skin that allows colonisation by invading microorganisms. In a person with diabetes, an impaired immune response is common, and this may predispose an ulceration to microbial invasion, with resultant damage to host tissues and an

Key Messages

- DFUs are a serious cause of morbidity and mortality and are associated with significant burdens to health care providers

- the role of microorganisms involved in causing infection in these wounds have primarily centred around planktonic microorganisms as causative agents in acute diabetic foot infections
- supporting data from researchers exploring if DFUs are complicated by biofilm have been sparse and are often reported using low sample sizes

inflammatory response that is characterised as a clinical infection (1). In some patients who receive optimal standards of care (including off-loading, regular sharp debridement and re-vascularisation) and who do not exhibit overt clinical infection, failure of the DFU to heal might be explained by the presence of biofilm. Planktonic microorganisms that are responsible for acute infections may be readily identified through cultivation-based approaches, while multi-species sessile communities of microorganisms or biofilms may not be detected by the same cultivation methods.

There is also a lack of diagnostic tests to define the presence of wound biofilm, and there are no quantifiable biomarkers. To augment clinical practice, some clinicians have used what they believe are 'clinical cues' of biofilm presence through naked eye observations (2–4). Such signs have included a shiny, translucent, slimy layer on the non-healing wound surface; the presence of slough or fibrin; and gelatinous material reforming quickly following removal in contrast to slough and other devitalised tissue or fibrin, which often take longer to reform. As biofilms are microscopic in nature, doubt has been expressed as to whether biofilms can be visually observed by clinicians. Unfortunately, chronic wound clinical observations have not been cross-correlated to microscopy approaches, which are better suited for defining the presence of biofilm.

The primary objectives of this study were to visualise and confirm the presence of biofilm in DFUs and better understand if they consist of mono- or multi-species biofilms. Secondary objectives were to ascertain if commonly used clinical cues were accurate in detecting wound biofilm. SEM, FISH/PNA-FISH and next-generation DNA sequencing were utilised to answer these objectives.

Methods

Patient population

In this prospective study, 65 consecutive patients aged over 18 years presenting to the Liverpool Hospital High Risk Foot Service with a DFU were enrolled over a 6-month period. Individuals were eligible for the study if they had either a DFU that had not responded to standard care and were not healing within an appropriate timeframe (i.e. chronic DFU) or presented with a DFU (acute or chronic DFU) and a new acute clinical infection as defined by the Infectious Disease Society of America Guidelines for Diabetic Foot Infection (5). Tissue biopsies were obtained from the wound edge for each participant after cleansing the wound with NaCl 0·9%. Clinical observations of DFUs were recorded for each patient. Ethics approval for this study was granted by the South West Sydney Local Health District Research and Ethics Committee (HREC/14/LPOOL/487, SSA/14/LPOOL/489).

Specimen collection, storage and next-generation DNA sequencing workflows

Specimen collection, storage and the work flows for performing DNA extraction and next-generation DNA sequencing were performed as previously described (6).

Fluorescent in situ hybridisation (FISH) and peptide nucleic acid-based fluorescence in situ hybridisation (PNA-FISH)

Biopsy material was embedded in an optimal cutting temperature (OTC) embedding matrix (Fisher Scientific, Waltham, MA), frozen at −80°C, cryo-sectioned to a thickness of 6 μm and mounted on SuperFrost Plus slides (Menzel-Glaser, Lomb Scientific, Sydney, Australia). Different types of probes were utilised for in situ hybridisation as previously described by Thurnheer (7). The choice of specific probes was based on DNA sequencing results that allowed the identification of the major genera/species of interest to target. The genus-specific probe Cy3 labelled *Staphylococcus* spp. probe (final concentration 5 ng/µl) (8), Fluor 488 labelled *Pseudomonas* spp. specific probe (final concentration 20ng/µl) and a universal bacterial probe Fluro 488 or Cy3 (final concentration 5 ng/ul) (9) were employed. For PNA-FISH, probes and kits were sourced commercially (AdvanDx, Inc., Woburn, MA) using previously described methods (10). Briefly, species-specific *Staphylococcus aureus*/coagulase-negative *Staphylococci* (CNS) probes were used in conjunction with universal bacterial probe. The hybridisation solution was added drop-wise to each tissue section and hybridised at 55°C for 90 minutes. The slides were washed for 30 minutes at 55°C in wash solution. Once dry, the coverslip was mounted using a single drop of mounting medium. Slides were examined using CLSM (Zeiss Axio Imager Microscope and/or ZEISS LSM 880, Carl Zeiss Ltd., Herefordshire, UK). Images were processed using ZEISS ZEN Imaging Software (black edition) and Imaris v 8·4, ImarisXT, Bitplane.

Scanning electron microscopy (SEM) and image interpretation

DFU biopsy samples were fixed in 3% glutaraldehyde, followed by three washes of 0·1M phosphate buffer prior to serial ethanol dehydration and hexamethyldisilazane incubation (Polysciences, Inc., Warrington, PA) as described previously (11). Dried samples were coated with 20-nm gold film in a sputter coater and examined in a scanning electron microscope. Each sample was scored based on the amount of bacteria/biofilm observed using an arbitrary 5-point scale as previously reported (12). Each tissue sample was viewed under SEM, averaging 2 hours per sample. Tissue was screened for microbial aggregates and extracellular polymeric substances (EPS) from the wound surface downwards, working in a zigzag pattern at magnifications ranging from ×300 to approximately ×5500.

Characterisation and visualisation of DFU biofilm

The presence or absence of biofilms in DFUs was confirmed through SEM or FISH/PNA-FISH. For the purpose of the study, the definition of biofilm was 'microbial aggregates surrounded by a self-produced or host-derived matrix adhering to natural or artificial surfaces in the host, or aggregates associated with but not directly adherent to the surface' (13).

Clinical wound observations

Wound observations at the time of presentation were collected based on previous assumptions of 'clinical cues' relating to the presence of biofilm. These were the presence of slough, excessive exudate, poor-quality granulation tissue, presence of pyocyanin, gelatinous material on the wound surface and gelatinous material that reforms quickly.

Statistics

Data relating to the presence or absence of DFU biofilm and clinical wound observations were tested using non-parametric methods (binomial probabilities). The hypothesis for the presence of biofilm was based on a previous report that 60% of chronic wounds have biofilm (14), and this was set as the expected proportion. For clinical wound observations, no previous data were available. Expected proportions were set at 50%, that is, no more than chance alone of clinical wound observations being positive when biofilm was found to be positive through microscopy. Data were analysed through Statistical Package for Social Sciences Version 23 (SPSS Inc., Chicago, IL). For all comparisons and modelling, the level of significance was set at $P < 0{\cdot}05$. Data are given as mean, median and standard deviation (±).

Results

Over a 12-month study period, 65 consecutive patients with DFUs were recruited. Study demographics are reported briefly. The majority of patients were male (49, 74·2%), and there were 17 female (25·8%) patients. The mean age of study subjects was 58·5 years (±12·3). Type 2 diabetes predominated (type 2 = 58, 87·9%, type 1 = 7, 10·6%), and the mean duration of diabetes was 13·9 years (±7·3). Clinically infected DFUs were present in 40 patients (60·6%). These were subdivided by duration of the DFU prior to the development of a new acute infective episode: short-duration DFU (<6 weeks) with new acute infection (7, 17·5%) and chronic DFU >6 weeks with new acute infection (33, 82·5%). The remaining patients with DFUs (26, 39·4%) were classified as chronic non-healing with no acute clinical infection.

Microscopy analysis

The presence of biofilm was visualised and confirmed in all samples (65 of 65, 100%) using either SEM, FISH/PNA-FISH or both ($P = 0{\cdot}0001$) (Table 1). Multiple images were viewed under microscopy for each sample to provide an overall score. SEM images identified a predominance of coccoid cells, which often appeared to be coated with EPS (Figure 1). When scoring samples, the majority had large micro-colonies (approximately 100 cells) plus the presence of continuous or thick film of extracellular matrix, that is, a score of 4 (52%) or 5 (36%). Biofilm presence was negative in two samples by SEM (S19 and S48), and a further seven SEM samples were not obtained due to inadequate amounts of DFU tissue. In the absence of SEM, all samples were positive using PNA-FISH with CLSM. DFUs were further sub-categorised for biofilms structures based on their duration, with most samples coming from chronic DFUs (>6 weeks with or without infection, 60 of 65, 92%). Five DFUs of short duration (<6 weeks) with clinical infection were also visualised as containing biofilm.

The spatial organisation of microorganisms was explored using PNA-FISH techniques and identified dense microbial aggregates (biofilm) (Figure 2). Generally, biofilms were not present in a uniform manner across the entire wound bed. Sampled tissue sections with species-specific and universal bacterial probes revealed areas of biofilm that were solely mono-species (Figure 3A) or multi-species biofilms (Figure 3B). We also identified areas of combinations where both mono-species and multi-species were located within the same sampled tissue section (Figure 3C).

Clinical wound observations

Using binomial probabilities, the probability of clinical observations associated with the positive presence of biofilm through microscopy were explored (Table 2). Except for excessive exudate, the probability of clinicians accurately identifying biofilm using clinical observations was no better than chance alone.

Next-generation DNA sequencing

The microbiome of DFUs was explored through next-generation DNA sequencing. Microorganisms contributing greater than 10% relative abundance per individual DFU sample are reported at the genera and species level where possible (Figure 4). The most abundant bacteria were (in rank order) *Staphylococcus* spp. *(S. aureus, S. epidermidis), Corynebacterium* spp. *(C. straitum, C. simulans), Streptococcus agalactiae, Anaerococcus* spp. *(Peptostreptococcus anaerobius), Peptoniphilus* spp., *Pseudomonas* spp. *(Pseudomonas auerginosa) and Prevotella* spp. *(P. melaninogenica, P. nanceiensis).*

Discussion

Employing a suite of microscopy and molecular approaches to analyse DFU tissue specimens, we identify the presence of densely aggregated colonies (both mono- and multi-species) of bacteria often surrounded by an extracellular matrix in tissue biopsies from 65 DFUs. This represents the largest data set in the literature and supports the view that biofilms are ubiquitous in DFUs and play host to a diverse ecology.

The clinical significance of our findings suggests that biofilms may have a pathogenic role across a spectrum of DFU presentations. We identified biofilm in three different wound states: short-duration DFUs (<6 weeks) with acute infection,

Table 1 Biofilm analysis and DFU location, wound duration in weeks and whether infected or non-infected. Presence (+) or absence (–) of biofilm as determined by SEM and FISH analysis, degree of biofilm infection (score) and predominant species identified using massively parallel DNA sequencing

	Biofilm(+ or –)				Wound metrics		
Patient number	SEM	Score	FISH/PNA-FISH	Biofilm diversity	Location of DFU	Duration of DFU (weeks)	Infection status
S01	+	4	+	Multi-species only	Plantar metatarsal head	8	Infected
S02	+	5	+	Mono-species and multi-species	Plantar metatarsal head	16	Infected
S03	+	4	NS	NS	Plantar metatarsal head	36	Infected
S04	+	4	+	Mono-species and multi-species	Plantar metatarsal head	14	Infected
S05	+	3	NS	NS	Plantar metatarsal head	24	Infected
S06	+	4	NS	NS	Fifth toe dorsal	12	Infected
S07	+	4	+	Multi-species only	Plantar metatarsal head	72	Infected
S08	NS	NS	+	Mono-species only	Fourth toe dorsal	6	Infected
S09	+	4	+	Multi-species only	Second toe apex	8	Infected
S10	+	3	NS	NS	Heel	20	Infected
S11	+	4	NS	NS	Heel	12	Infected
S12	+	3	+	Mono-species and multi-species	Plantar metatarsal head	24	Infected
S13	+	4	+	Mono-species and multi-species	Plantar metatarsal head	6	Infected
S14	+	4	NS		Plantar metatarsal head	20	Infected
S15	NS	NS	+	Multi-species only	Fourth toe apex	12	Infected
S16	NS	NS	+	Mono-species and multi-species	Hallux	8	Infected
S17	+	4	+	Multi-species only	Hallux	26	Infected
S18	+	4	+	Mono-species only	Plantar metatarsal head	32	Infected
S19	–	0	+	Mono-species and multi-species	Hallux	12	Infected
S20	+	3	+	Multi-species only	Second toe apex	16	Infected
S21	+	5	+	Mono-species and multi-species	Medial hallux	8	Infected
S22	NS	NS	+	Mono-species and multi-species	Medial hallux	18	Infected
S23	NS	NS	+	Mono-species and multi-species	Heel	12	Infected
S24	+	4	NS	NS	Heel	24	Infected
S25	NS	NS	+	Multi-species only	Hallux apex	9	Infected
S26	+	5	+	Multi-species only	Plantar midfoot	3	Infected
S27	+	5	+	Mono-species and multi-species	Plantar metatarsal head	3	Infected
S28	+	5	NS	NS	Plantar metatarsal head	6	Infected
S29	+	5	NS	NS	Plantar midfoot	52	Infected
S30	+	4	NS	NS	Plantar midfoot	30	Infected
S31	+	5	NS	NS	Plantar metatarsal head	3	Infected
S32	+	5	NS	NS	Plantar metatarsal head	5	Infected
S33	+	4	+	Mono-species and multi-species	Heel	5	Infected
S34	+	5	+	Multi-species only	Heel	12	Infected
S35	+	5	+	Mono-species and multi-species	Heel	6	Infected
S36	+	4	NS	NS	Plantar metatarsal head	12	Infected
S37	+	4	NS	NS	Hallux	9	Infected
S38	+	4	+	Mono-species and multi-species	Hallux	8	Infected
S39	+	4	+	Multi-species only	Hallux	12	Infected
S40	+	4	NS	NS	Heel	72	Non-infected
S41	+	4	NS	NS	Plantar metatarsal head	40	Non-infected
S42	+	4	+	Mono-species and multi-species	Heel	6	Non-infected
S43	+	4	NS	NS	Plantar metatarsal head	24	Non-infected
S44	NS	NS	+	Mono-species and multi-species	Plantar metatarsal head	12	Non-infected
S45	+	4	+	Mono-species and multi-species	Heel	36	Non-infected
S46	+	5	+	Mono-species and multi-species	Plantar metatarsal head	72	Non-infected
S47	+	5	+	Multi-species only	Heel	7	Non-infected
S48	–	1	+	Multi-species only	Plantar midfoot	28	Non-infected
S49	+	4	+	Multi-species only	Heel	18	Non-infected
S50	+	5	+	Multi-species only	Heel	28	Non-infected
S51	+	4	+	Mono-species and multi-species	Plantar metatarsal head	27	Non-infected
S52	+	5	+	Mono-species and multi-species	Plantar metatarsal head	28	Non-infected
S53	+	4	NS	NS	Hallux	6	Non-infected
S54	+	5	+	Mono-species and multi-species	Hallux	6	Non-infected
S55	+	4	+	Mono-species and multi-species	Heel	6	Non-infected
S56	+	3	+	Multi-species only	Plantar metatarsal head	16	Non-infected
S57	+	5	+	Multi-species only	Heel	20	Non-infected

Table 1 Continued

	Biofilm(+ or −)				Wound metrics		
Patient number	SEM	Score	FISH/PNA-FISH	Biofilm diversity	Location of DFU	Duration of DFU (weeks)	Infection status
S58	+	5	+	Mono-species and multi-species	Lateral leg	14	Non-infected
S59	+	4	+	Multi-species only	Heel	10	Non-infected
S60	+	5	+	Mono-species and multi-species	Plantar metatarsal head	27	Non-infected
S61	+	4	+	Multi-species only	Hallux	8	Non-infected
S62	+	4	+	Mono-species and multi-species	Plantar metatarsal head	12	Non-infected
S63	+	5	+	Mono-species and multi-species	Hallux	6	Non-infected
S64	+	5	NS	NS	Heel	52	Non-infected
S65	+	5	+	Mono-species and multi-species	Plantar metatarsal head	9	Non-infected

NS, inability to obtain an additional sample for microscopy from that patient.

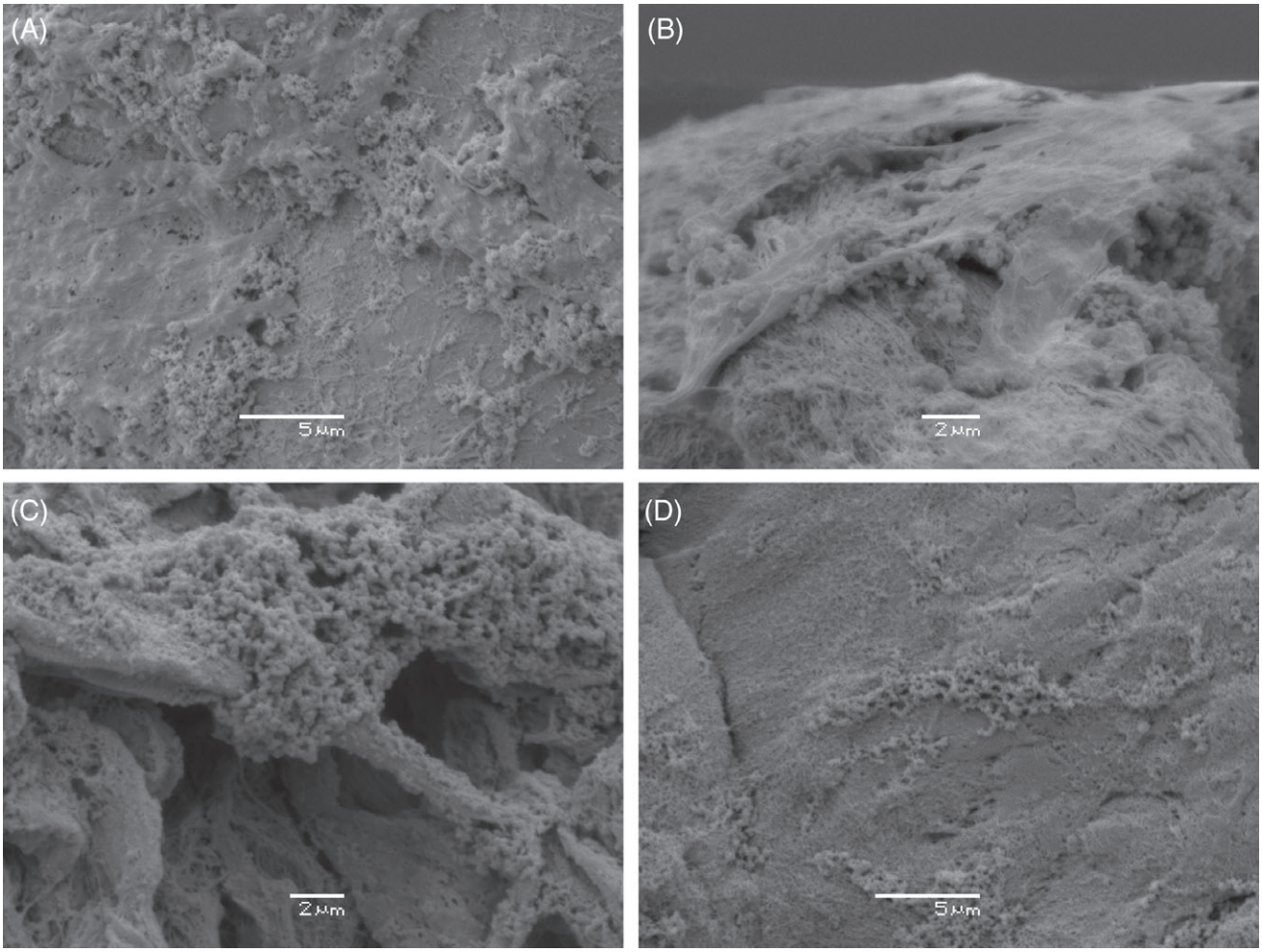

Figure 1 Scanning electron micrograph of DFU obtained from four patients demonstrating biofilm structure. (A) and (B) show large micro-colonies of predominantly coccoid microbial cells encased in thick extracellular matrix (EPS) and anchored to collagen bundles within the wound (biofilm score 5). (C) shows large micro-colonies of predominantly coccoid cells covered in a thin film of EPS (biofilm score 4). (D) Shows large micro-colonies but less EPS (biofilm score 3).

chronic DFUs with acute infection and chronic DFUs with no infection but are non-healing. The visualisation of biofilms in chronic non-healing wounds is as expected, where they have been proposed as a likely cause of wound chronicity (15). The exact mechanisms of biofilm impairment on the healing processes of wounds remain unclear. In vitro observations suggest the wound is kept in a vicious inflammatory state, preventing the normal wound healing cycles to occur (16–18). Recently, data by James and colleagues proposed a concept of localised low-oxygen tensions contributing to wound chronicity (19). Using oxygen microsensors and transcriptomics (examining microbial metabolic activities) to study in situ

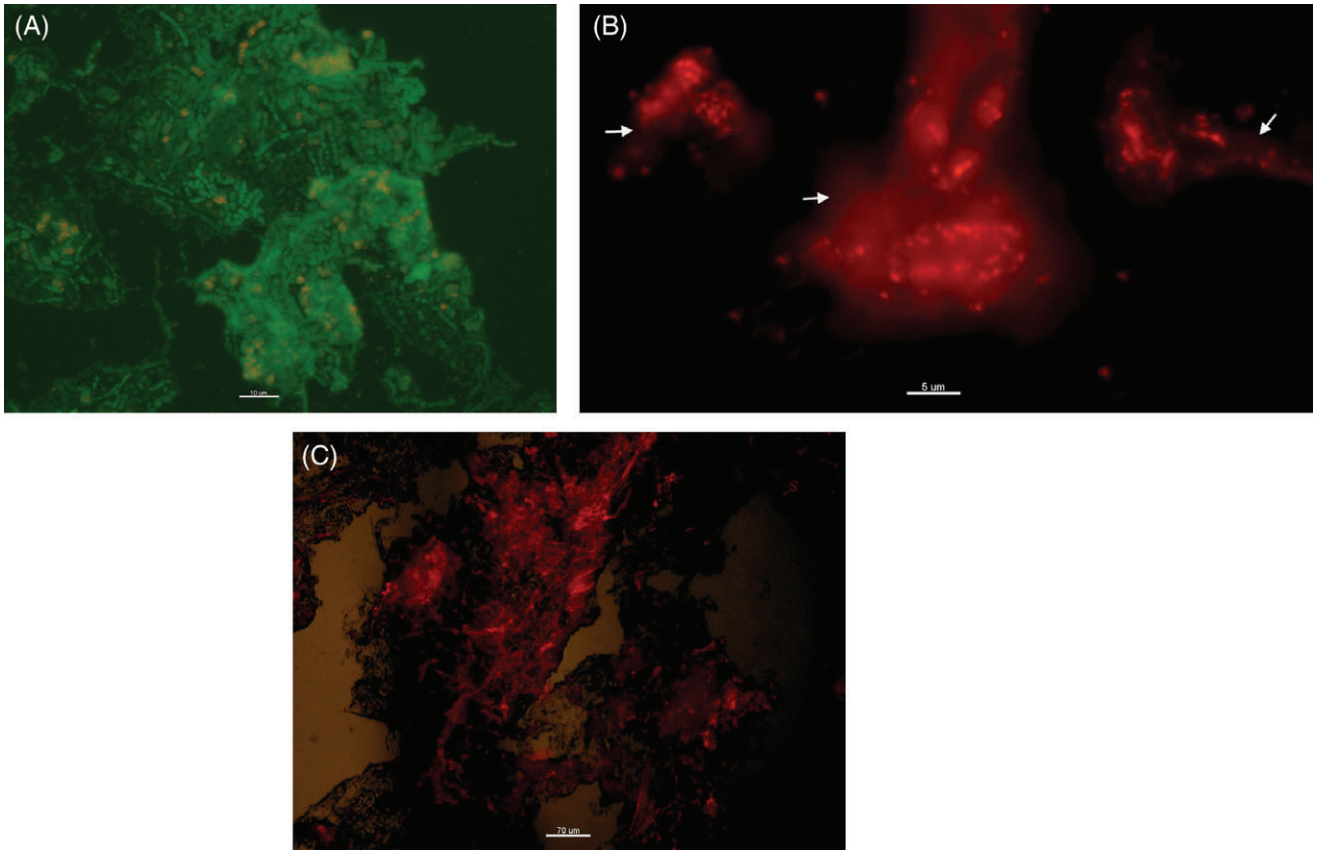

Figure 2 CLSM of biofilm demonstrated via FISH and PNA-FISH. (A) Patient 20, FISH with CLSM shows predominantly Gram-negative rods in biofilm using green-fluro-488-labelled probe targeting *Pseudomonas* spp. and yellow-Cy3-labelled universal bacterial probe. (B) Patient 4, PNA-FISH with CLSM illustrates different bacterial morphologies of a multi-species biofilm using fluorescent-labelled universal PNA probes. (C) Patient 48, FISH with CLSM using red-fluro-488-labelled universal probe is viewed at low magnification and illustrates the total amount of microbial biofilm on the tissue.

biofilms, James and colleagues identified steep oxygen gradients and induced oxygen-limitation stress responses from bacteria.

The presence of biofilm, however, in wounds of short duration (<6 weeks) presenting with an acute infection is less commonly reported. Five DFUs of short duration were captured in this study (range from 2 to 5 weeks) with biofilm being visualised in all patients. The general consensus is that biofilms are not responsible for acute infective episodes, for which planktonic microorganisms are the major driver (20). People who develop DFUs may be at increased risk of the earlier formation of biofilm. This may be explained by several ill-defined immunological deficits attributed to underlying hyperglycaemia (21) that contributes to a poor response of neutrophils to colonising or invading microorganisms (22) or from impairments in microbial phagocytosis (23). Although the number of samples to draw a valid conclusion is small ($n = 5$), it is unclear whether this phenomenon is specific to DFUs or can be observed in other chronic wound types and presents an interesting trend that warrants further exploration.

In this study, all the presenting infected DFUs had biofilm, but given that most DFUs were chronic at presentation, we would expect biofilm to be present but not necessarily involved as an acute pathogen of infection. It is plausible that the biofilm acts a reservoir for pathogens, and biofilm dispersal increases the presence of pathogenic planktonic microorganisms (24). To support this idea, the most abundant bacteria identified using DNA sequencing in this study was *S. aureus*. Species-specific probes for *S. aureus* used in our PNA-FISH analysis also confirmed *S. aureus* as being present in the clear majority of samples as dense microbial aggregates. In the absence of direct biofilm culture assays from our clinical isolates, we could refer to the plethora of evidence for *S. aureus*' profound ability to form biofilm, particularly on human skin and tissue (25). Furthermore, *S. aureus* has long been cited as the most common pathogen of infection in diabetic foot infections from culture-dependent studies (26), and we also identify *S. aureus* as being the predominant pathogen of infection in the presence of visualised biofilm in this study. It is also possible that the acute infections of chronic DFUs were caused by a new invasion of planktonic bacteria rather than dispersal from biofilm colonies.

When analysing the community structure of DFUs, multi-species communities comprising of both strict anaerobes and aerobic species were identified. Biofilms can form 'microniches' (27), with steep oxygen gradients occurring through biofilm or areas of altered pH or nitrate (28). These

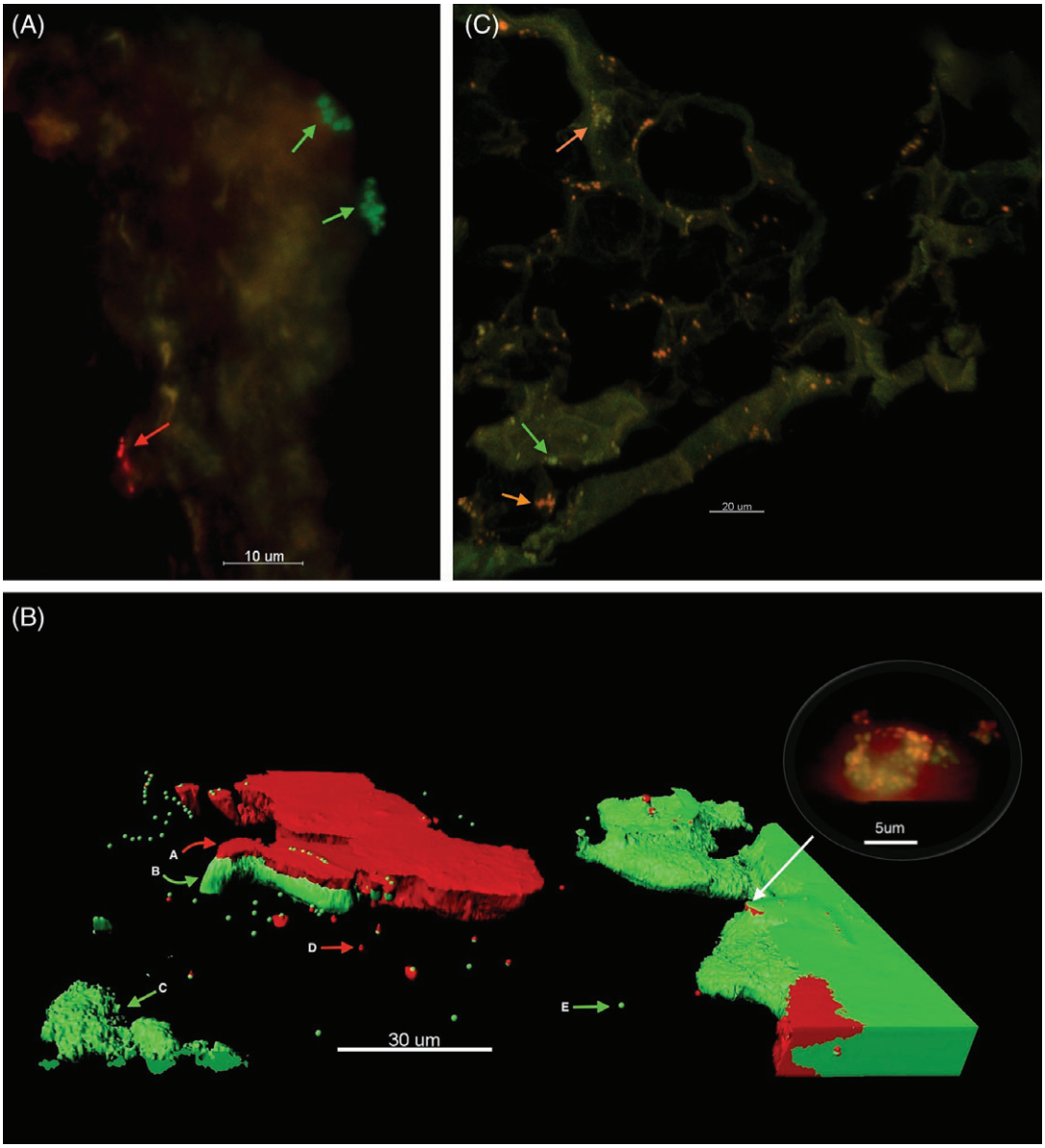

Figure 3 FISH and PNA-FISH with CLSM technique to explore the spatial organisation of microbial aggregates in DFU samples. (A) Patient 18, identifies two mono-species biofilm in the same wound, bacteria labelled with *S. aureus* PNA probe (green bacteria) and bacteria labelled with coagulase-negative *Staphylococci* (red). (B) Patient 27, three-dimensional view of a DFU biopsy depicted using the Imaris software. This highlights the structural complexity of biofilms where multi-species biofilm coexist with mono-species biofilms and planktonic microorganisms. (A) coagulase-negative *Staphylococci* is red-labelled PNA probe with (B) *S. aureus*, a green-labelled PNA probe. (C) Mono-species of *S. aureus* biofilm. (D) Planktonic aggregates of coagulase-negative *Staphylococci*. (E) Planktonic aggregates of *S. aureus*. Top right corner viewing bubble demonstrates standard CLSM view of multi-species biofilm under high magnification. (c). Patient 35, FISH with CLSM. Green arrow shows mono-species biofilm (*Staphylococcus* spp. specific probe), and orange arrow shows mixed-species biofilms (universal bacterial red probe).

studies confirm that distinct microniches exist at different depths in biofilms and thus make it possible to understand how metabolically diverse microorganisms coexist. While aerobic Gram-positive cocci were predominant through samples, several strict anaerobes were also present in the majority of samples, particularly *Clostridales family XI* members *Anaerococcus* spp., *Peptoniphilus* spp. and *Finegoldia* spp. Using culture-independent approaches, this group of fastidious bacteria have been previously reported as colonising DFUs in greater abundance when compared against laboratory-based culture techniques (29).

The clinical significance of having multi-species biofilms consisting of metabolically diverse microorganisms (i.e., aerobic and anaerobic microorganisms) is not clear, and there is no direct evidence to suggest patients with multi-species biofilms have less favourable outcomes than those with mono-species biofilms. Previous reports in the literature, however, have identified the occurrence of synergism between metabolically

Table 2 The probability that a clinical wound observation is related to the presence of biofilm†

		Binomial test				
		Category	*N*	Observed prop.	Test prop.	Exact Sig. (two-tailed)
Presence of slough	Group 1	Yes	38	0·58	0·50	0·215
	Group 2	No	27	0·42		
	Total		65	1·00		
XS Exudate	Group 1	No	5	0·08	0·50	0·000*
	Group 2	Yes*	60	0·92		
	Total		65	1·00		
Poor tissue quality	Group 1	No	29	0·45	0·50	0·457
	Group 2	Yes	36	0·55		
	Total		65	1·00		
Signs of pyocyanin	Group 1	No*	52	0·80	0·50	0·000*
	Group 2	Yes	13	0·20		
	Total		65	1·00		
Gelatin Wound Surface	Group 1	No*	46	0·71	0·50	0·001*
	Group 2	Yes	19	0·29		
	Total		65	1·00		
Gelatin Reforms Quickly	Group 1	No*	53	0·82	0·50	0·000*
	Group 2	Yes	12	0·18		
	Total		65	1·00		

* *P* value < 0·05.

†The binominal probability questions asked here is a yes or no response. Therefore, statistical significance should be denoted by an * if the visual marker is accurate in detecting biofilm. The benchmark was set at 50% occurrence rate for the visual marker to be present. The results below indicate that visual markers (with exception of XS exudate) are no better than chance alone.

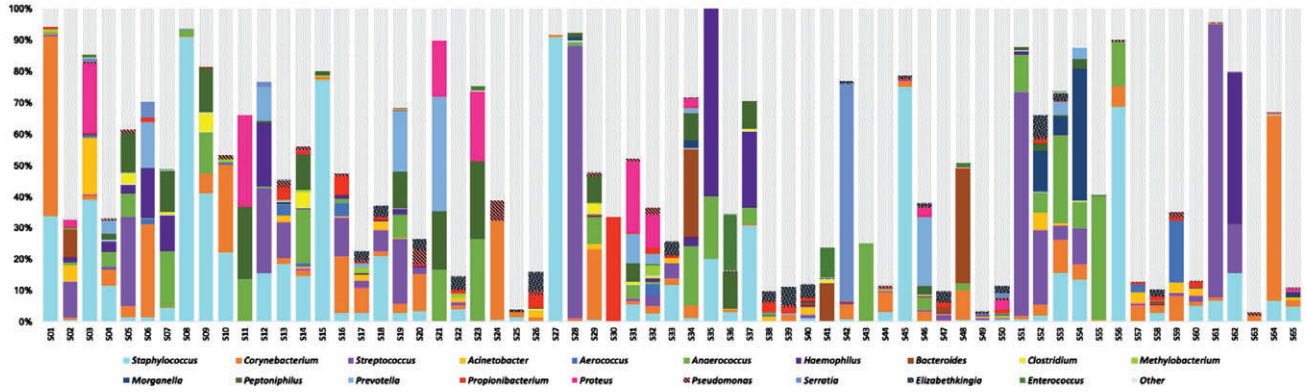

Figure 4 Next-generation DNA sequencing of 65 DFUs. Bar graphs depict the most common genera of microorganisms in DFUs. The vertical axis refers to relative abundance across DFUs. Horizontal axis is the participant number.

diverse microorganisms that demonstrate a greater pathogenicity/virulence and or an enhanced tolerance to therapeutics (30). As with most chronic wounds complicated by biofilm, their tolerance to many forms of treatments that include systemic antimicrobials, topical antiseptics and disinfectants is well-documented (31,32). This has led to expert groups promoting multi-faceted biofilm-based wound-care approaches (13) to tackle these tolerant phenotypes.

Part of this biofilm-based approach is the use of systemic antimicrobials in the presence of clinical infection. A question, therefore, that needs to be explored is whether clinicians need to consider altering systemic antimicrobial therapy based on the presence of multi-species biofilms containing additional 'hidden' anaerobes? Most clinicians with access to local and international antimicrobial stewardship guidelines and guidelines specifically for diabetic foot infection are guided to use empirical first-line antimicrobials that provide a broad spectrum of activity against anaerobes (such as Amoxicillin and Clavulanic acid). These guidelines also promote the use of antimicrobials with further targeted anaerobe action (such a Metronidazole). In this instance (and except for biofilm tolerance to antibiotics), most anaerobes are likely targeted by conventional regimens.

One assumption when exploring the microbiome of chronic wounds complicated by biofilm using DNA sequence techniques is that the polymicrobial nature of these wounds must, in turn, equal multi-species biofilms (33). This is not the case. We identify cases where *S. aureus* forms a mono-species biofilm next to a neighbouring multi-species biofilm. This suggests a non-random distribution of microbial biofilms where mono-species biofilm could form in multi-species infections. This scenario has been previously documented by Bjarnsholt

and colleagues using PNA-FISH on chronic wound samples (34). They reported that many microbial aggregates were mono-microbial and belonged to either *P. aeruginosa* or *S. aureus* (identified using specific probes). Additionally, the depth and location of these microbial aggregates was correlated to the depth of the wound bed. *S. aureus* were primarily located close to the wound surface, whereas *P. aeruginosa* was primarily located deeper in the wound bed. The authors concluded that microbial aggregates function in a non-random distribution.

In a recent study on visualising wound biofilms, the clinical observation of a gel-like substance/film was then correlated to biofilm presence through microscopy (35). The study concluded that 10 of the 16 samples revealed recurring wound bed film and that this sign was indicative of macroscopic biofilm presence. In contrast, for 26 samples analysed in this study, except for excessive exudate, the probability of a clinician accurately identifying biofilm using clinical observations is not better than chance alone. Furthermore, wounds that exhibit a gel-like substance where biofilm is confirmed through microscopy might have this only in the presence of specific biofilm species as not all wounds exhibit this feature (an example of this could be the mucoid *P.aeruginosa*, which produces the viscous polysaccharide alginate in cystic fibrosis). We propose that clinical cues are not useful for detecting biofilm presence in DFUs, but larger sample sizes from both DFUs and other chronic wound aetiologies are required to verify our results.

In conclusion, microscopy visualisation, when combined with molecular approaches, confirms biofilms are ubiquitous in DFUs, and a paradigm shift of managing these complicated wounds needs to consider anti-biofilm strategies.

Acknowledgements

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KJ, MM, SJ, IG, HD, HH and KV contributed equally to this work.

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Effect of cadexomer iodine on the microbial load and diversity of chronic non-healing diabetic foot ulcers complicated by biofilm *in vivo*

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Objectives: The performance of cadexomer iodine was determined against microbial populations from chronic non-healing diabetic foot ulcers (DFUs) complicated by biofilm *in vivo*, using molecular, microscopy and zymography methods.

Methods: Chronic non-healing DFUs due to suspected biofilm involvement were eligible for enrolment. DNA sequencing and real-time quantitative PCR was used to determine the microbial load and diversity of tissue punch biopsies obtained pre- and post-treatment. Scanning electron microscopy and/or fluorescence *in situ* hybridization confirmed the presence or absence of biofilm. Zymography was used to determine levels of wound proteases.

Results: Seventeen participants were recruited over a 6 month period. Scanning electron microscopy and or fluorescence *in situ* hybridization confirmed the presence of biofilm in all samples. Eleven participants exhibited $\log_{10}$ reductions in microbial load after treatment (range 1–2 $\log_{10}$) in comparison with six patients who experienced <1 $\log_{10}$ reduction ($P = 0.04$). Samples were tested for levels of wound proteases pre- and post-treatment. Reductions in the microbial load correlated to reductions in wound proteases pre- and post-treatment ($P = 0.03$).

Conclusions: To the best of our knowledge, this study represents the first *in vivo* evidence, employing a range of molecular and microscopy techniques, of the ability of cadexomer iodine to reduce the microbial load of chronic non-healing DFUs complicated by biofilm. Further analyses correlating log reductions to optimal duration of therapy and improvements in clinical parameters of wound healing in a larger cohort are required.

Introduction

In a person with diabetes, foot ulceration leaves a physical break in the protective barrier of the skin. Factors including a retarded host immune response and pathogen-related dynamics (such as virulence or pathogenicity) may predispose the diabetic foot ulcer (DFU) to further planktonic microbial replication and invasion. This may result in damage to host tissues and an inflammatory response that is characterized as a clinical infection.[1]

Increasing evidence into the role of microorganisms involved in DFUs (and other wound aetiologies) has identified that single free-floating microorganisms (planktonic) that are responsible for acute infections, may not necessarily represent the ecology of microorganisms present in chronic non-healing wounds. Instead, the focus has orientated towards the concept of biofilms, which differ markedly in both their physiology and activity. The exact mechanisms of biofilm impairment on the healing processes of wounds are not clear, but general consensus suggests that biofilms maintain an elevated inflammatory state within the wound.[2]

Microorganisms in biofilm exhibit enhanced tolerances to chemical, biological and host attack compared with those in planktonic forms. *In vitro* biofilm models have demonstrated that microbial biofilms can withstand antimicrobial concentrations 100–

1000 times higher than that of their planktonic counterparts.[3–6] This may go towards explaining why some wounds fail to heal with standard care and why chronic infections persist.[7] *In vitro* models assessing the effectiveness of many antimicrobials used in wound-related products have identified that these treatments often have variable and poor results against microbial cells in biofilm phenotypes.[8–10]

One potential explanation for this treatment failure is because the wound care treatments do not target or are ineffective against biofilm. Cadexomer iodine, however, has demonstrated superior efficacy against microbial biofilms when tested *in vitro* and in animal models, when compared against other topical antimicrobials used in wound care dressings.[8–10]

Therefore, as a pilot study massively parallel DNA sequencing, real-time quantitative PCR (qPCR), microscopy techniques [scanning electron microscopy (SEM) and fluorescence *in situ* hybridization (FISH)] and gel zymography were used to identify if the topical antimicrobial cadexomer iodine could reduce the microbial load of chronic non-healing DFUs complicated by biofilm *in vivo*. Additional interests were to explore the effects of cadexomer iodine on the microbial communities pre- and post-treatment, and determine the levels of wound proteases [matrix metalloproteinase (MMP)-2 and MMP-9]. It was not the intention of this pilot study to correlate any effects of microbial loads or diversity to wound healing outcomes.

Materials and methods

Ethics

Ethics approval for this study was granted by the South West Sydney Local Health District Research and Ethics Committee (HREC/14/LPOOL/487, SSA/14/LPOOL/489).

Exclusion criteria

Patients with clinical signs of infection as per the IDSA guidelines,[1] known osteomyelitis that was associated with the DFU, or patients who had received any topical or systemic antimicrobial therapy 2 weeks prior to enrolment, were not eligible. The reason for excluding patients with clinical infection was the assumption that these wounds would be driven predominantly by planktonic microorganisms.[11] Participants with general contraindications to the use of cadexomer iodine as per the manufacturer's guidelines were also excluded.[12]

Subjects and sample collection

Individuals were eligible for the study if they had a chronic non-healing DFU defined as a wound of >6 weeks duration failing to respond to standard care.[13] Cadexomer iodine was applied every second day over a 7 day treatment period (total of three applications). Sharp debridement of DFUs was withheld over the 7 day treatment period given this was likely to impact the results significantly.[14] A tissue biopsy was obtained from the wound edge for each participant before and after treatment. All tissue samples were frozen at −80°C until completion of the last patient and processed in bulk so as to reduce any bias. Additionally, we collected broad demographics and wound metrics. Primary endpoint was a reduction in microbial load 7 days post-treatment. Secondary analysis included the exploration of community richness and diversity of DFUs pre- and post-treatment, visual changes to biofilm structures and alteration to levels of wound proteases (MMP-2 and MMP-9).

Cadexomer iodine (Iodosorb®, Smith & Nephew)

Iodosorb® ointment is designed as a carrier system enabling the delivery of iodine, which can penetrate the cell wall of microorganisms and disrupt protein and nucleic acid structure and synthesis.[15] Iodosorb® consists of small polysaccharide beads (cadexomer) containing 0.9% iodine, which, in the presence of wound exudate, causes the polysaccharide beads to swell allowing a slow sustained release of iodine into the wound.

Specimen collection and storage

A 3 mm (width)×10 mm (depth) tissue punch biopsy was obtained from the edge of each DFU after cleansing the wound with 0.9% NaCl. Tissue biopsy samples were obtained for all participants at baseline and day 7. For SEM, an additional 2 mm (width) × 10 mm (depth) was necessary. Following removal, tissue samples were rinsed vigorously in a PBS bath to remove any coagulated blood and to reduce the number of planktonic microorganisms. Samples were cut transversely into two 1.5 mm pieces for DNA sequencing and FISH. DNA samples were immediately placed into RNA*later*® (Ambion, Inc.) for 24 h at 4°C and then frozen at −80°C until DNA extraction. Tissue samples for FISH were immediately fixed in 4% paraformaldehyde overnight at 4°C, then transferred into PBS and frozen at −80°C. Tissue samples for SEM were immediately fixed in 3% glutaraldehyde overnight at 4°C, then transferred into 0.1% PBS and frozen at −80°C. All tissue samples remained at −80°C until study completion to reduce any bias and were processed in bulk.

Tissue processing workflow

DNA extraction

Five to ten milligrams of human chronic DFU biopsy samples was defrosted on ice prior to DNA extraction. Genomic DNA was extracted using a Mo Bio PowerBiofilm DNA isolation kit (Mo Bio cat. no. 24000–50) following the manufacturer's instructions.

Next-generation DNA sequencing to determine bacterial diversity

Massively Parallel DNA sequencing was carried out by a commercial laboratory (Australian Centre for Ecogenomics) targeting the V4 region of the 16S rRNA gene using eubacterial universal primers 515F (TCGTCGGCAGCGT CAGATGTGTATAAGAGACAGCCAGCAGCYGCGGTAAN) and 806R (GTCTCG TGGGCTCGGAGATGTGTATAAGAGACAG GGACTACHVGGGTWTCTAAT).

Preparation of the 16S library was performed as described at the Australian Centre for Ecogenomics using the workflow outlined by Illumina. In the first stage, PCR products of ∼466 bp were amplified according to the specified workflow with an alteration in polymerase used to substitute Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, MA, USA) in standard PCR conditions. Resulting PCR amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). Purified DNA was indexed with unique 8 bp barcodes using the Illumina Nextera XT 384 sample Index Kit A-D (Illumina FC-131–1002) in standard PCR conditions with Q5 Hot Start High-Fidelity 2X Master Mix. Indexed amplicons were pooled together in equimolar concentrations and sequenced on the MiSeq Sequencing System (Illumina) using paired end sequencing with V3 300 bp chemistry.

Sequence analysis and quality control

Reads in FASTQ format were imported to CLC genomics workbench version 8.5.1 using the microbial genome finishing module (CLC bio; Qiagen, Aarhus, Denmark), for sequence quality control and analysis. Workflows for sequence quality control and operational taxonomic units (OTUs) clustering were based on previously reported wound microbiome analysis.[16] OTUs were defined as molecular proxies for describing organisms based on their

phylogenetic relationships to other organisms, and were reported at either the genera or species level identification where possible.

Briefly, after sequence and quality control measures, reads were assigned to OTUs using SILVA[17] at 99% similarity at the genus level and species level where possible. OTUs were aligned using MUSCLE[18] to reconstruct a phylogenetic tree, allowing the estimation of the alpha diversity pre- and post-treatment for each DFU. This included both community richness (rarefaction) and community diversity (Shannon–Weaver Index). Rarefaction curves allow the estimation of the number of unique microbial taxa within a sample and the Shannon–Weaver Index is a measure of diversity that includes the number of unique microbial taxa and their relative evenness within each sample. Thus, a higher Shannon–Weaver Index correlates to a greater diversity.

qPCR to determine microbial load in DFU biofilms

We utilized qPCR to determine microbial load in DFU biofilms, as previously reported.[19,20] To quantify the total amount of bacterial DNA/mg of tissue in each wound tissue sample we obtained the copy number of the 16S rRNA gene (copies/μL), which was normalized against the human 18S rRNA gene (copies/μL) in each chronic wound sample.

Characterization and visualization of DFU biofilm

The presence or absence of biofilms in chronic non-healing DFUs was confirmed through SEM or FISH. For the purpose of this study, we used definitions promoted by an expert group to characterize biofilm as being (i) microbial aggregates surrounded by a self-produced or host-derived matrix adhering to natural or artificial surfaces in the host, or (ii) aggregates associated with, but not directly adherent to, the surface.[21]

FISH. Two to three millimetres of DFU tissue was embedded in optimal cutting temperature (OCT) embedding matrix (Tissue-Plus™ O.C.T Compound, Thermo Fisher Scientific, Waltham, MA, USA) and frozen at −80°C overnight. DFU tissues were sectioned to a thickness of 6 μm and mounted on SuperFrost Plus slides (Thermo Fisher Scientific). For visualization of microorganisms and biofilm, confocal laser scanning microscopy was combined with FISH. The hybridization process used was previously described by Thurnheer *et al.*[22] Briefly, two different probes were utilized for *in situ* hybridization: Fluor 488-labelled universal probe[23] (final concentration 5 ng/μL); and Cy3-labelled *Staphylococcus* spp.-specific probe (final concentration 5 ng/μL).[24] All images were examined under confocal laser scanning microscopy (Carl Zeiss Ltd, Herefordshire, UK). All images were processed using ZEISS ZEN Imaging Software (black edition).

SEM and image interpretation

In vivo microbial biofilms associated with DFU tissue were sampled at 5–200 μm for optimal visualization through SEM.[25] Two to 3 mm of DFU tissue was fixed in 3% glutaraldehyde, followed by three washes of 0.1 M PBS prior to serial ethanol dehydration and hexamethyldisilazane (Polysciences, Inc., Warrington, PA, USA), as described previously.[20] Dried samples were coated with 20 nm gold film and examined using SEM. Each sample was scored based on the amount of bacteria/biofilm observed using an arbitrary five-point scale as previously reported:[26] score 0 = no bacteria observed; 1 = single individual cells; 2 = small microcolonies (∼10 cells); 3 = large microcolonies (∼100 cells); 4 = continuous film; and 5 = thick continuous film.

Determination of wound proteases

Wound fluids were collected from DFUs pre- and post-treatment and stored at −80°C for quantification of MMPs (MMP-2 and total MMP-9) by gel band zymography, as previously described.[27]

Table 1. Patient demographics and wound metrics at baseline

Patient demographics	
male	11 (65%)
female	6 (35%)
type 1 diabetes	2 (12%)
type 2 diabetes	15 (88%)
mean age in years	66 (±13.6)
Wound metrics	
location of ulcer	
plantar metatarsal head	8
calcaneum	3
dorsal foot	2
ankle	2
hallux	2
duration of ulcer in weeks	
mean duration of ulcer at baseline	25 (±20.7, range 6–72)
University of Texas wound classification	
1A	10 (59%)
1C	1 (5.8%)
2A	5 (29.4%)
2C	1 (5.8%)
size of ulcer	
mean DFU size at baseline	3.7×2.7 cm (L × W)

Statistics

Wound metrics and microbiome data were analysed through Statistical Package for Social Sciences Version 23 (IBM, Armonk, NJ, USA). Wilcoxon signed-rank tests for paired samples of non-parametric data were performed on pre- and post-treatment microbial $\log_{10}$ reductions. χ^2 was used to correlate OTUs and Shannon–Weaver indices to $\log_{10}$ reduction. One-way ANOVA was used to estimate variances between MMP levels before and after treatment. Logistic regression was employed to correlate microbial reduction to MMP levels. CLC genomics workbench version 8.5.1 in combination with the microbial genome-finishing module (CLC bio; Qiagen) was used to analyse DNA sequence data. For all comparisons and modelling, the level of significance was set at $P < 0.05$. Data are given as mean, standard deviation (±) and 95% CI.

Results

A total of 17 patients with chronic non-healing DFUs were enrolled. One ulcer from each patient was biopsied before and after treatment. Patient demographics and wound metrics are shown in Table 1. Microbial load was determined via qPCR for all 17 DFU samples pre- and post-treatment. However, three samples were removed from exploring community richness (rarefaction) and diversity (Shannon–Weaver Index) due to a low number of 16S rRNA gene sequence reads, leaving 12 samples available for analysis. In total (for these 12 samples), 703346 high-quality DNA sequences were generated (before = 384772 and after- = 318574), with a median of 31452 per sample level data (range 1137–61820). The clustering of OTUs identified 1976 unique taxa from which low abundance OTUs were removed (<0.1%), leaving 368 OTUs for further analysis. Only eight wound fluid samples were available for analysis. Protein concentrations in the remaining samples were too low for analysis.

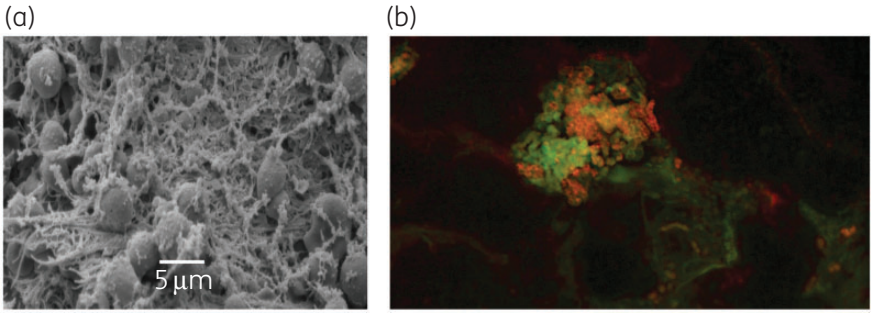

Figure 1. (a) SEM from a sample pre-treatment. Microbial aggregates with self-produced or host-derived matrix that we describe as a stringy-like glue appearance. SEM imaged at 5 μm. (b) FISH image depicts microbial aggregates of mixed species biofilm [Fluor 488-labelled universal bacterial probe (red) and Cy3-labelled *Staphylococcus* spp.-specific probe].

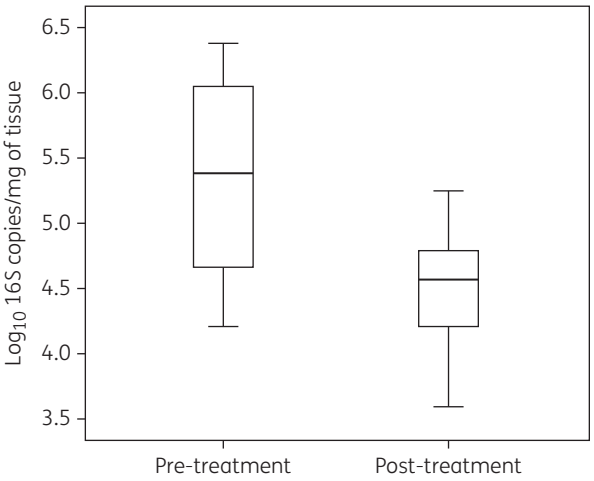

Figure 2. Effect of cadexomer iodine pre- and post-treatment. Boxplots show median and IQR, with whiskers showing the range of $\log_{10}$ values of all 17 patients.

Confirmation of the presence or absence of biofilms in each DFU

The presence of biofilm was visualized and confirmed in all 17 participants using SEM, FISH or both (Figure 1a and b). Biofilm architecture was graded (via SEM) using an arbitrary sliding scale previously reported.[26] The median value of DFU biofilm architecture reduced between pre- and post-treatment samples; pre-treatment median was 4 (large microcolonies ∼100 cells and a continuous film/matrix) and the post-treatment median was 3 (large microcolonies ∼100 cells).

Reduction in microbial load of chronic non-healing DFUs complicated by biofilm

The application of cadexomer iodine resulted in 11 samples achieving up to and greater than a 1 $\log_{10}$ reduction (mean microbial load pre-treatment = 5.92 $\log_{10}$ 16S copies/mg of tissue versus 4.56 $\log_{10}$ 16S copies/mg of tissue, $P = 0.02$, 95% CI 3.43 to 4.61 $\log_{10}$) (Figure 2). Six samples had no change or increases in $\log_{10}$ (mean microbial load pre-treatment = 5.22 $\log_{10}$ 16S copies/mg of tissue versus 5.20 $\log_{10}$ 16S copies/mg of tissue).

Analysis of community richness and diversity of chronic non-healing DFUs treated with cadexomer iodine

The most abundant OTUs (based on the number of DNA sequences represented when pooling samples) are noted in Figure 3. The richness and diversity of chronic non-healing DFUs pre- and post-treatment are also reported at the individual sample level of microorganisms contributing to >5% of abundance in rank order (Table 2). Post-treatment identified diversity shifts with increasing environmental contaminants. These microorganisms are identifiable only by molecular methods (in the majority), and included in the Proteobacteria-E01-9C-26 Marine Group, Proteobacteria-ARKDMS-49, Archaea-*Cenarchaeum*, *Elizabethkingia* spp., Bacteroidetes-*Rhodothermacae* and Proteobacteria-*Rhodothalssium*. Furthermore, some of these microorganisms are known extremophiles, existing in hostile, niche environments.

The taxonomic richness and diversity of pre- and post-treatment pooled samples identified no differences between mean OTUs (pre-treatment = 115, SD = 69.3 versus post-treatment = 112, SD = 74.3, $P = 0.53$) and mean Shannon–Weaver indices (pre-treatment = 2.8, SD 1.2 versus post-treatment = 3, SD = 1.4, $P = 0.58$). When exploring this at the individual sample level there was large heterogeneity between pre- and post-treatment OTUs and Shannon–Weaver indices. To ascertain if the number of unique OTUs and their relative abundance were correlated to reductions in $\log_{10}$ values, a χ^2 test was performed. Reductions in $\log_{10}$ did not correlate to reductions in either OTUs ($P = 0.85$) or Shannon–Weaver indices ($P = 0.72$).

MMP levels pre- and post-treatment

The mean total MMP-9 and MMP-2 levels in eight samples reduced following 7 days application of cadexomer iodine (Figure 4). However, only total MMP-9 reached statistical significance (mean total MMP-9 before = 2202 mg/10 μL versus mean total MMP-9 after = 1065 mg/10 μL, 95% CI −14.5 to 2287, $P = 0.05$). Mean MMP-2 before = 181.9 versus mean MMP-2 after = 89 (95% CI −61.3 to 246.7, $P = 0.197$). In general, any reductions in the levels of MMPs were correlated to reductions in the microbial load ($P = 0.03$).

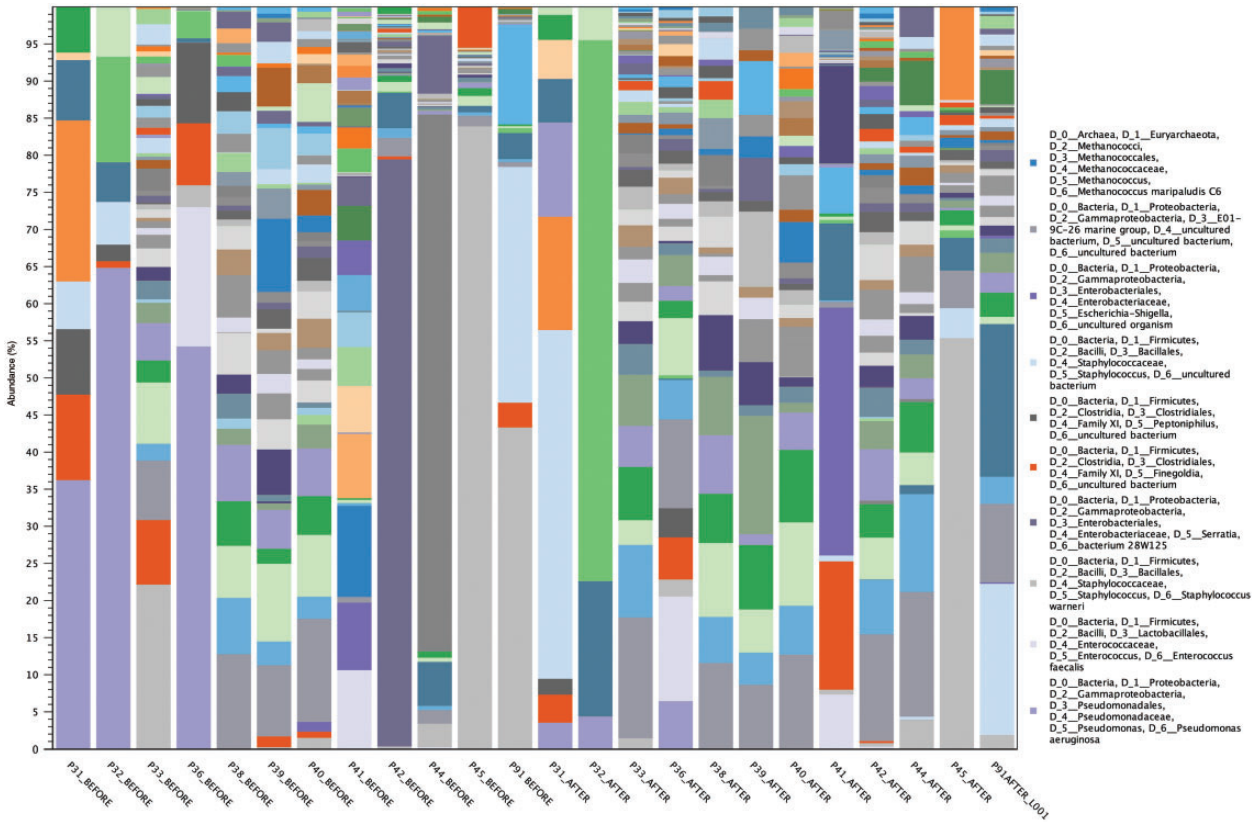

Figure 3. Most frequent microorganisms based on the pooling of all DNA sequences and the total number of DNA sequences attributed to each microorganism. This graph also exemplifies the contrast between low- and high-frequency taxa at the genus/species level. High-frequency taxa consist of a few predominant microorganisms (typically between 1 and 9 microorganisms; samples 31, 32, 36, 42, 44, 45 and 91) and low-frequency taxa consist of multiple microorganisms (typically 10-fold in comparison with high-frequency taxa; >30–90 microorganisms; samples 33, 38, 39, 40 and 41).

Discussion

Using a combination of DNA sequencing, qPCR, SEM and FISH, the ecology of wounds and presence of microbial biofilms was explored. To the best of our knowledge, we are the first to employ these suites of molecular and microscopy techniques to show that cadexomer iodine can reduce the microbial load of chronic non-healing DFUs complicated by biofilm. We also show that in reducing microbial load, concomitant reductions in wound proteases are also achieved.

Massively parallel DNA sequencing allowed the exploration of chronic non-healing DFU microbiome, and provided useful insights that these wounds support complex polymicrobial communities. Molecular methods also demonstrated that cadexomer iodine had a broad level of antimicrobial activity in reducing both facultative anaerobes such as *Staphylococcus* spp., *Serratia* spp., *Pseudomonas* spp. and obligate anaerobes including *Clostridiales family XI*.

Both facultative and obligate anaerobes were detected together in chronic non-healing DFU samples positive for biofilm presence. Microelectrode and transcriptomic analysis of *in vitro* biofilms have identified that the interior of biofilms house niche areas of altered pH levels, oxygen and nutrient depletion.[3,28] Oxygen in particular is noted for its depletion at the substratum layers of biofilms and in the centre of microcolonies, explaining why wounds complicated by biofilm can harbour such diverse microorganisms. This may indirectly support the action of cadexomer iodine against biofilms, as it suggests penetration to deeper areas that house obligate anaerobes is possible, as identified by 16S rRNA of pre- and post-treatment reductions in obligate anaerobes.

Using a combined molecular and microscopy approach can provide an extended understanding of the effects of antimicrobials on wounds. This approach for example provided insights into how cadexomer iodine affected microbial populations. Using sample level data, *Serratia* spp. were identified as contributing to 75% of abundance pre-treatment and 0% post-treatment in sample 42 (Figure 5a). This also correlated with a >1 $\log_{10}$ reduction in microbial load as determined by qPCR. Similarly, for sample 44, *P. aeruginosa* contributed to 88% of abundance pre-treatment and 0.3% post-treatment, with a 0.76 $\log_{10}$ reduction in microbial load (Figure 5b). This suggests that cadexomer iodine was effective in reducing the abundant microorganisms in these cases.

In contrast, there was no $\log_{10}$ reduction in microbial load for sample 45 (pre-treatment $= 4.56^5$ and post-treatment $= 4.73^5$). While community profiling revealed a minor reduction in

Staphylococcus spp. (Figure 6a), this was countered with a concomitant increase in other microorganisms. In this case, FISH was able to demonstrate the lack of action against the bulk of microorganisms in sample 45, with pre- and post-treatment images depicting the presence of microbial aggregates as biofilm in chronic non-healing DFU tissue samples (Figure 6b).

In five patients, no $\log_{10}$ reduction was noted with the use of cadexomer iodine. Considering the accepted notion that biofilms are tolerant to antimicrobials, and combining the potential attributes of extremophile (and non-extremophile) microorganisms, this may explain why some wound microorganisms were tolerant to treatment. Post-treatment microbiome analysis identified that these microorganisms increased in abundance from previously low numbers, whereas skin flora microorganisms such as *Pseudomonas* spp. decreased in abundance following treatment (indicating cadexomer iodine were effective). This diversity shift may have occurred as nutrient availability increased or where mutual benefit arose.[29]

Table 2. Pre- and post-treatment community diversity in microorganisms contributing to >5% abundance of each individual sample

Genera	Samples	Average abundance (%)	Aerotolerance
Pre-treatment			
Pseudomonas spp.	5	58.5	aerobe
E01-9C-26 Marine	4	11.2	unknown
Staphylococcus spp.	4	58	facultative
Rhodothermaceae spp.	3	5	unknown
Finegoldia spp.	3	7.8	anaerobe
Elizabethkingia meningoseptica	3	6.3	aerobe
Cornyebacterium spp.	3	5.3	aerobe
Peptoniphilus spp.	2	9.5	anaerobe
Ananerococcus spp.	2	9.2	anaerobe
Proteus penneri	2	20.5	facultative
Post-treatment			
E01-9C-26 Marine	8	10	unknown
ARKDMS49	7	5.7	unknown
Cenarchaeum	5	5.1	unknown
Cyanobacteria—subsection I	5	7.4	unknown
Rhodothermaceae spp.	4	5.8	unknown
Rhodothalassium spp.	3	7	unknown
Cornyebacterium spp.	3	11	aerobe
Elizabethkingia meningoseptica	3	6.6	aerobe
Staphylococcus spp.	3	42.8	facultative
Proteus penneri	2	39	facultative

Study limitations

The primary aim of this study was to ascertain the effects of cadexomer iodine on microbial populations associated with the presence of biofilm, however without the controlled conditions afforded by *in vitro* biofilm models we are not able to fully rule out that planktonic microorganisms contaminated samples. Our rationale to provide a strong argument that planktonic microorganisms were of a negligible proportion of microbial cells, with the bulk of cells being biofilm, is supported by our visualization techniques using SEM and FISH. These methods identified significant aggregates of microbial cells with extracellular polymeric substance (EPS). Furthermore, we vigorously rinsed all tissue samples during preparation as a method to reduce planktonic microorganisms not adhered to tissue. Alternative methods to reduce planktonic microorganisms from contaminating biofilm models have only been alluded to from *ex vivo* animal explants,[10] with no reports of this approach on human tissue.

To measure total microbial load, qPCR was utilized as the technique of choice, with previous wound-related PCR studies adopting this methodology.[30,31] We acknowledge with this approach the inability of qPCR (based on 16S rRNA gene) to

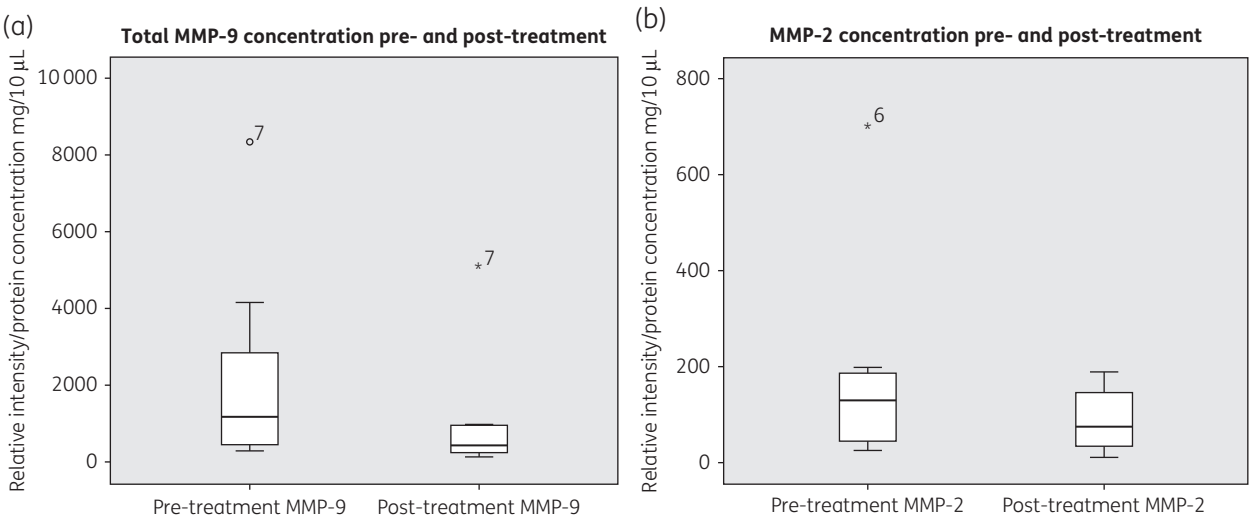

Figure 4. (a) Total MMP-9 values pre- and post-treatment. (b) MMP-2 values pre- and post-treatment. Boxplots show the median and IQR, with whiskers showing the range apart from outliers (>1.5×IQR).

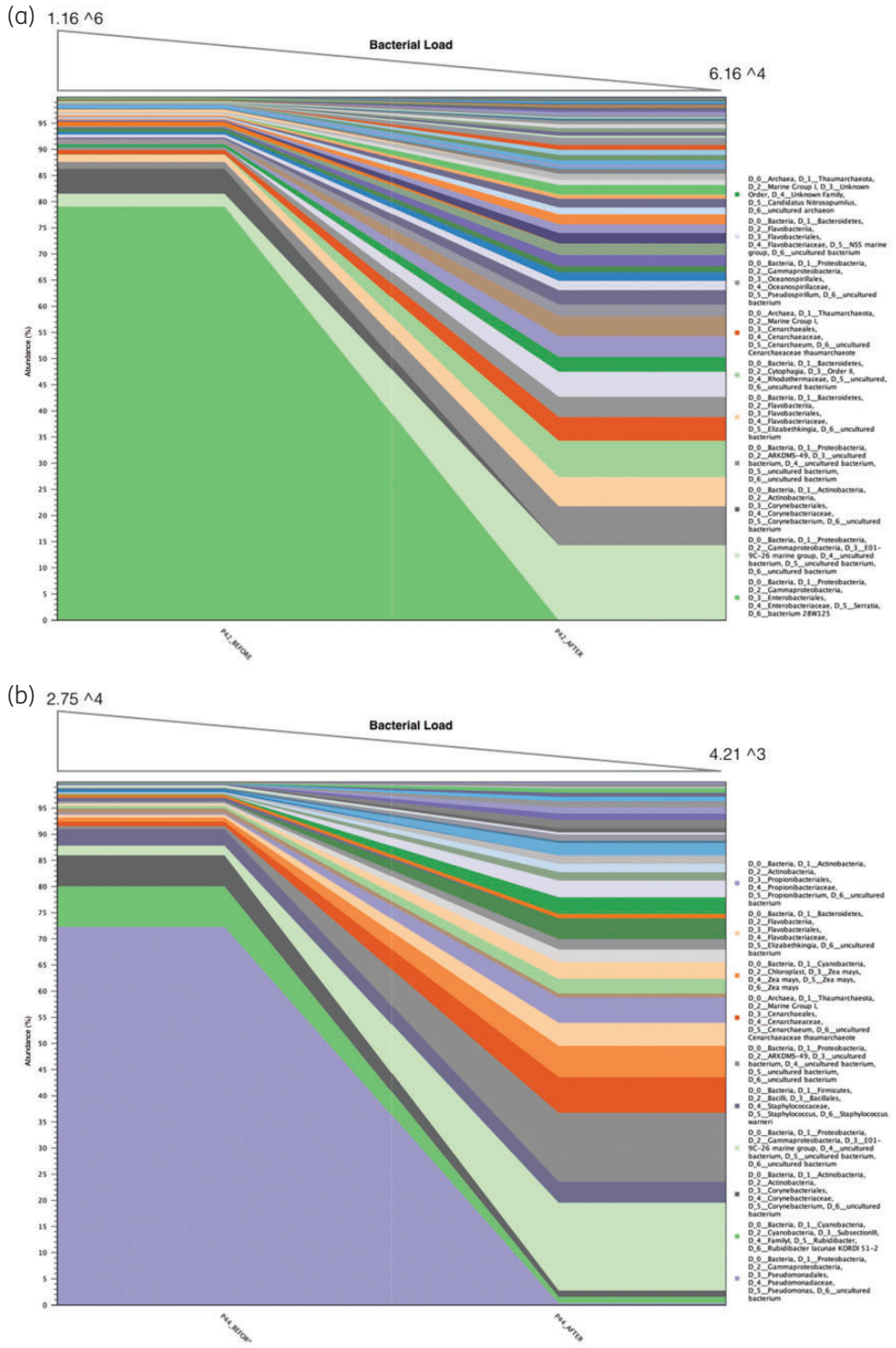

Figure 5. Pre- and post-treatment community diversity identified through 16S rRNA sequencing. (a) In the left-hand side, sample 42 identifies *Serratia* spp. contributing to 75% abundance pre-treatment, and in the right-hand side, this reduces to 0% post-treatment. Top of the graph identified the reduction in microbial load by over 1 $\log_{10}$ reduction determined through qPCR (99% reduction in microorganisms). Therefore, the left-hand pane is a reflection of the remaining 1% of microorganisms. (b) Sample 44 identified *Pseudomonas* spp. contributing to 88% abundance pre-treatment and 0.3% post-treatment with a 0.83 $\log_{10}$ reduction.

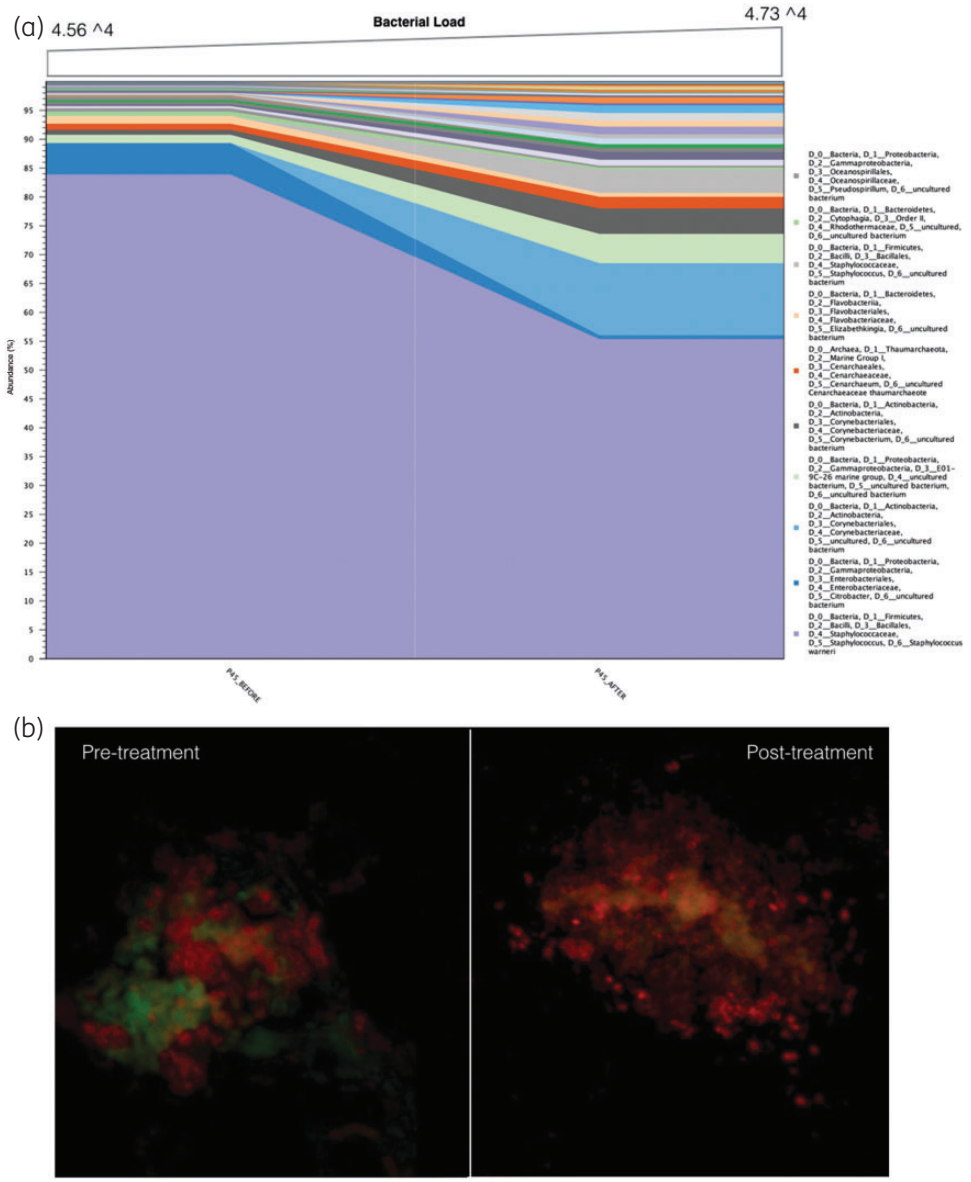

Figure 6. (a) Sample 45 identifies a small reduction in *S. aureus*. This is countered by a concomitant increase in other microorganisms allowing an increase in community diversity, but not overall microbial load. (b) This lack of action is also identified through FISH. Red colour represents a universal probe (Fluor 488) and the green represents a species-specific probe for *S. aureus* (Cy3). Some reduction in *S. aureus* aggregates post-treatment is noted, but the biofilm remains in bulk.

differentiate live or dead bacteria. Other commonly employed techniques such as molecular viability testing (pre-RNA analysis or RT-qPCR) are able to detect live or dead microorganisms. The problem with using these techniques for our diabetic ulcer samples is the predominance of biofilm phenotype cells present in the tissue. Pre-RNA rapidly responds to nutrition stimulation and target metabolism, not actual microbial load. Given that biofilm cells have low metabolism in comparison with planktonic cells, this may lead to variations in the 16S pre-RNA level between the biofilm and planktonic cells and different growth conditions.[32] The log reductions noted in this study therefore represent the minimal response and we acknowledge that some of the bacteria detected by qPCR could be dead, resulting in a lower calculated efficacy for cadexomer iodine.

Lastly, only eight samples with adequate protein concentration were available for analysis. In the majority, obtaining enough wound fluid post-treatment was difficult, which may be associated with reduced wound inflammation with reductions in microbial loads. Alternatively, the time of dipstick applications were undertaken during 30 min appointments and this may not have been long enough to allow the wounds to leak adequately onto the three dipsticks required.

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Transparency declarations

M. M. received a consultancy fee from Smith & Nephew to undertake work not associated with this study, but pertaining to producing educational material on biofilms in chronic wounds. All other authors: none to declare.

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Evaluation of short exposure times of antimicrobial wound solutions against microbial biofilms: from *in vitro* to *in vivo*

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Objectives: Test the performance of topical antimicrobial wound solutions against microbial biofilms using *in vitro*, *ex vivo* and *in vivo* model systems at clinically relevant exposure times.

Methods: Topical antimicrobial wound solutions were tested under three different conditions: (*in vitro*) 4% w/v *Melaleuca* oil, polyhexamethylene biguanide, chlorhexidine, povidone iodine and hypochlorous acid were tested at short duration exposure times for 15 min against 3 day mature biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*; (*ex vivo*) hypochlorous acid was tested in a porcine skin explant model with 12 cycles of 10 min exposure, over 24 h, against 3 day mature *P. aeruginosa* biofilms; and (*in vivo*) 4% w/v *Melaleuca* oil was applied for 15 min exposure, daily, for 7 days, in 10 patients with chronic non-healing diabetic foot ulcers complicated by biofilm.

Results: *In vitro* assessment demonstrated variable efficacy in reducing biofilms ranging from 0.5 $\log_{10}$ reductions to full eradication. Repeated instillation of hypochlorous acid in a porcine model achieved <1 $\log_{10}$ reduction (0.77 $\log_{10}$, $P = 0.1$). Application of 4% w/v *Melaleuca* oil *in vivo* resulted in no change to the total microbial load of diabetic foot ulcers complicated by biofilm (median $\log_{10}$ microbial load pre-treatment = 4.9 $\log_{10}$ versus 4.8 $\log_{10}$, $P = 0.43$).

Conclusions: Short durations of exposure to topical antimicrobial wound solutions commonly utilized by clinicians are ineffective against microbial biofilms, particularly when used *in vivo*. Wound solutions should not be used as a sole therapy and clinicians should consider multifaceted strategies that include sharp debridement as the gold standard.

Introduction

Chronic wounds are a serious cause of morbidity and mortality, and are associated with reduced patient health-related quality of life. The impacts on healthcare providers are reflected in the staggering cost of managing these wounds and associated comorbidities, with £5.3 billion attributed to UK National Health Service expenditure.[1] Increasing evidence on the microorganisms involved in chronic wounds has identified that planktonic cells may not necessarily represent the phenotypic behaviour of microorganisms involved in chronic non-healing wounds. The focus has shifted towards the concept of microbial aggregates (biofilms), which differ markedly in their phenotypic behaviour and may contribute to the delayed healing of wounds.[2] In addition, the ecology of chronic wounds explored through molecular DNA-based technologies (and not cultivation-based methods) has identified these wounds to be complicated by complex polymicrobial communities.[3]

Once established, complex biofilm communities often become highly tolerant to standard treatment and removal/eradication paradigms, yielding several hallmark features that distinguish biofilm phenotypes from those of planktonic counterparts. The most notable of these is a remarkable tolerance to antimicrobial agents,[4,5] and host immune defences.[6] The increasing awareness and promotion of the biofilm concept within the wound care arena has led to a dramatic rise in the use of topical antimicrobial solutions as part of wound care therapeutics.[7]

Unfortunately, the evidence for use of particular topical antimicrobials in the treatment of biofilm-associated wounds is based on *in vitro* methodologies that lack standardization and clinical relevance to their intended applications.[8] For example, the antibiofilm effects of wound solutions, for which outcomes are based on reductions in biofilm markers (i.e. biomass, cfu/mL, LIVE/DEAD® stain viability), have been reported at exposure times far greater than their intended use. Many wound care/device companies promote a 15 min exposure time for their respective antimicrobial solutions (seconds for irrigation solutions), yet the bulk of data for effectiveness of these products *in vitro* have only reported outcomes at 24 h exposure times.[9–11] This has important consequences at the treatment level where clinicians often seek guidance from laboratory-based studies (owing to a lack of available *in vivo* data) in choosing the most relevant and effective agent to reduce microbial biofilms. Therefore, *in vitro* data based on greater exposure times may not reflect the most clinically appropriate outcomes for clinicians using these products at shorter exposure times. This is highlighted succinctly by Castaneda *et al.*,[12] who showed that in an *in vitro* biofilm model, antimicrobial susceptibility increased with antimicrobial exposure time.

The present study was designed to explore whether shorter durations of exposure to antimicrobial wound solutions were effective against microbial biofilms: (i) *in vitro* against mature biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*; (ii) in an *ex vivo* porcine skin explant model against mature *P. aeruginosa* biofilms; and (iii) *in vivo* in 10 patients with chronic non-healing diabetic foot ulcers (DFUs).

Materials and methods

Bacteria

The biofilm-forming reference strains utilized *in vitro* were *S. aureus* [ATCC® 25923™ (MSSA)] and *P. aeruginosa* (ATCC® 25619™), and *P. aeruginosa* PA01 (ATCC® BAA-47™) was used in the *ex vivo* porcine skin explant model.

Antimicrobial wound solutions

The solutions examined, any incorporated antimicrobials/antiseptics and their respective manufacturers, were as follows: surfactant-based antiseptic solution with 4% w/v *Melaleuca* oil (SBMO; Woundaid® Woundwash; Mundipharma, Singapore); surfactant-based antimicrobial solution with polyhexamethylene biguanide (SBPHMB; Prontosan®; B. Braun Medical, Melsungen, Germany); superoxidized solution (SOS) containing sodium hypochlorite, hypochlorous acid, sodium chloride and oxidized water (Microcyn®; Oculus Technologies of Mexico); chlorhexidine (CHX) 4.5 mg/30 mL (0.015% w/v) and cetrimide 45 mg/30 mL (0.15% w/v) irrigation solution (Pfizer, New York, USA); povidone iodine antiseptic solution 10% w/v equivalent to 1% w/v available iodine (PVP-I; BETADINE®; Mundipharma, Singapore); NaCl 0.9% (Baxter, IL, USA).

The decision to use SOS for the *ex vivo* porcine explant model and SBMO for the human *in vivo* study was based on clinical relevance. Both the use and promotion of these 'newer generation' solutions with antimicrobial properties (as opposed to traditional antimicrobials of CHX and PVP-I) by clinicians and industry for action against wound biofilm has increased significantly over the last decade. They now represent the predominant products used for wound cleansing and debridement.

Experimental models

In vitro model

Biofilm, containing 10^7–10^8 cells/coupon of *P. aeruginosa* (ATCC 25619) and 10^6 cells/coupon of *S. aureus* ATCC 25923 was grown under shear (130 rpm) on polycarbonate coupons in a CDC biofilm reactor (BioSurface Technologies Corp., Bozeman, MT, USA) as previously described by our group,[13] in 400 mL of 15 g/L (50%) tryptic soy broth (Sigma–Aldrich, St Louis, MO, USA) at 35 °C in batch phase for 48 h, followed by incubation in fresh medium (20% tryptic soy broth, 6 g/L) for a further 24 h. Coupons were washed in 10 mL PBS to remove loosely attached planktonic bacteria. Each coupon had 10^7–10^8 *P. aeruginosa* or 10^6 *S. aureus*. Five antiseptic treatments were tested (SBMO, SBPHMB, SOS, CHX, PVP-I); four coupons were exposed to each treatment condition for 15 min, while an additional four coupons were used as controls.

The numbers of bacterial colony forming units (cfu) per coupon were tested in triplicate by sonication in an ultrasonic bath (Soniclean; JMR, Australia) for 10 min with a sweeping frequency of 42–47 kHz at 20°C. The coupon was then vortexed for 2 min in 2 mL of PBS followed by a sequential 10-fold dilution and plate count. Pre- and post-exposure average cfu/coupon was expressed as $\log_{10}$. Bacterial cell viability pre- and post-exposure was also assessed using *Bac*Light™ (LIVE/DEAD® Bacterial Viability Kit, 7012; Molecular Probes, Invitrogen, Carlsbad, CA, USA) in conjunction with confocal laser scanning microscopy (CLSM) and expressed as the percentage of viability as determined by Imaris (v8.4, ImarisXT, Bitplane). For CLSM, we used an inverted laser scanning confocal microscope (ZEISS LSM 880; Carl Zeiss Ltd, Herefordshire, UK) for all the samples, with oil-immersion lenses (63× and 100×) and acquisition parameters of: frame size, 1024×1024; speed, 6; averaging, 2; bit depth, 12.

Ex vivo porcine skin explant model

The *ex vivo* porcine skin explant biofilm model used in this study is previously described[14] and a detailed description can be found in the Supplementary data (Part S1, available as Supplementary data at *JAC* Online). One pig was used to obtain all explants, which were freshly harvested, shaved, cleaned and inflicted with a partial thickness excision wound. Explants were then sterilized by first submerging the explants in PBS containing 0.6% hypochlorous acid and 0.5% Tween 80 for 5 min then transferring them to a chlorine gas chamber for 45 min, followed by submerging the explants again in PBS containing 0.6% hypochlorous acid and 0.5% Tween 80 for 5 min. The sterile explants were rinsed twice in sterile PBS then transferred into 150 mm diameter by 25 mm deep culture plates (176 cm^2 surface area) (Corning 430599) containing 0.5% tryptic soy soft agar containing antibiotic (gentamicin at 50 mg/mL) to limit planktonic growth and promote biofilm growth on the explants. One hundred microlitres of *P. aeruginosa* PA01 (~10^7–10^8 cfu/mL) was inoculated onto the explants and incubated for 3 days at 37 °C. Porcine explants were subjected to three test groups: (i) negative pressure wound therapy alone (control); (ii) negative pressure wound therapy with instillation therapy for 12 cycles of 10 min of soak/dwell with SOS, totalling 24 h for the experiment; and (iii) negative pressure with instillation therapy for 12 cycles of 10 min of soak/dwell with saline (NaCl 0.9%). After 24 h, six 8 mm biopsies were obtained from the porcine skin explant and processed for measurement of cfu/mL and scanning electron microscopy (SEM). For each test group, six experiments were established and the cfu was averaged over these.

In vivo clinical study

We used a combined molecular and microscopy approach described previously[15] to better understand the effects of a topical antimicrobial solution against the microbial load and diversity of chronic non-healing DFUs complicated by biofilm (Supplementary data Part S2). Ten patients with chronic non-healing DFUs (and not on current antimicrobial therapy) were enrolled over a 6 month period from a tertiary referral hospital (Liverpool Hospital High Risk Foot Service, Liverpool, Sydney). Ethics approval for this study was granted by the South West Sydney Local Health District Research and Ethics Committee (HREC/14/LPOOL/487, SSA/14/LPOOL/489). Sterile gauze was soaked in SBMO and applied to the wound for 15 min, every day for 7 days. Sharp debridement of tissue was withheld over the 7 day treatment period, as this would likely have affected the primary outcome measure.[16] Tissue punch biopsies were obtained from the wound edge for each participant after cleansing the wound with NaCl 0.9% pre- and post-treatment. These were subjected to quantitative PCR (qPCR) to determine the total microbial load, next generation DNA sequencing to explore the microbiome of chronic DFUs and the effects on microbial communities following topical antimicrobial therapy, SEM to visualize biofilm structures and fluorescent *in situ* hybridization in conjunction with CLSM to examine spatial organization of microbial aggregates.

Statistics

Mann–Whitney *U*-tests were used to assess differences between pre- and post-$\log_{10}$ cfu using the Statistical Package for Social Sciences Version 23 (SPSS Inc., Chicago, IL, USA). CLC genomics workbench version 8.5.1 in combination with the microbial genome-finishing module (CLC bio, Qiagen Aarhus, Denmark) was used to analyse DNA sequence data. QIIME was utilized to visually represent data. Analysis of variance and permutational analysis of variance were used for statistical analysis of alpha and beta diversity measures. Principal coordinates analysis plots with Bray–Curtis distances were used to assess how dissimilar microbial communities were pre- and post-treatment. Community richness of DFUs was presented using richness index reporting the number of unique operational taxonomic units (OTUs) in each wound sample. The Shannon–Weaver Index is an ecological measure of diversity that includes the number of unique microbial taxa and their relative evenness within each sample. For all comparisons and modelling, the level of significance was set at $P < 0.05$. Data are given as mean, median ($\pm$SD) and 95% CI.

Results

Antimicrobial efficacies of topical wound solutions against mature biofilms in vitro

The effects of topical antimicrobial solutions on reductions in $\log_{10}$ cfu/coupon following treatments at 15 min and 24 h are shown in Figure 1(a and b). LIVE/DEAD® stain with CLSM and the percentage of red signal (dead/damaged cells) and green signal (viable cells) at 15 min exposures are noted in Supplementary data Part S3. At 15 min exposures PVP-I was the only solution to show complete and efficient killing of both *S. aureus* and *P. aeruginosa* biofilms (6 and 7 $\log_{10}$ reduction, $P = 0.001$). CHX was effective against *S. aureus* biofilms showing complete removal of all bacteria (6 $\log_{10}$ reduction, $P = 0.001$), and further demonstrated a 3.96 $\log_{10}$ cfu reduction against the *P. aeruginosa* biofilm ($P = 0.01$). In contrast, SOS demonstrated complete eradication of the *P. aeruginosa* biofilm (7 $\log_{10}$ reduction, $P = 0.001$) and a $\geq$4 $\log_{10}$ cfu/mL reduction against *S. aureus* (4.3 $\log_{10}$ reduction $P = 0.01$). No significant reduction in *S. aureus* counts was observed for treatment with SBPHMB (0.8 $\log_{10}$ reduction); however, it was highly effective against *P. aeruginosa* biofilm showing complete eradication (7 $\log_{10}$ reduction, $P = 0.01$). Treatment with SBMO was ineffective against both *S. aureus* and *P. aeruginosa* biofilm. In contrast, treatment of biofilm with topical antimicrobials for 24 h exposure showed complete and efficient killing of biofilm, except for SBMO, which failed to eradicate *S. aureus* (but still achieved a $\geq$2.5 $\log_{10}$ cfu/coupon).

Antimicrobial efficacy of SOS against mature biofilms in an ex vivo porcine skin explant model

Levels of *P. aeruginosa* PA01 viable bacteria after 12 cycles of negative pressure therapy and instillation of saline or SOS are shown in Figure 2. The total bacterial bioburden (planktonic + biofilm) growing on the porcine skin explant was 8.0 $\log_{10}$ cfu/mL, of which 7.1 $\log_{10}$ cfu/mL were biofilm bacteria, as defined by being tolerant to incubation in 50× MIC gentamicin for 24 h at 37 °C. When porcine skin explants with mature *P. aeruginosa* PA01 biofilm were exposed to 12 cycles of negative pressure wound therapy alone without instillation of any solution (control for negative pressure wound therapy), which is equivalent to 'pulsed or intermittent negative pressure wound therapy', the level of *P. aeruginosa* PA01 cfu was reduced to 6.9 $\log_{10}$ cfu/mL. When the porcine skin explants were treated with negative pressure wound therapy with 12 cycles of instillation with saline with a 10 min exposure time, the level of *P. aeruginosa* PA01 bacteria was the same (6.9 $\log_{10}$ cfu/mL). Changing the instillation solution to SOS and using the same 12 cycles of instillation, the level of *P. aeruginosa* PAO1 bacteria was essentially the same as with saline instillation, with 6.8 $\log_{10}$ cfu/mL surviving the instillation treatment. In contrast, planktonic and biofilm bacteria were completely eradicated using the *in vitro* CDC biofilm reactor laboratory test.

As shown in Supplementary data Part S4, SEM of the wound area in the porcine skin explants demonstrated very thick continuous biofilm on untreated explants (panel A). SEM of explants treated with saline instillation (panel C) or explants treated only with negative pressure and no instillation (panel D) showed a reduction in biofilm structures, but substantial amounts of attached bacteria were still present. Explants treated with SOS instillation (panel B) also showed a reduction in the biofilm structure but persistence of attached bacteria.

The effect of SBMO against the microbial load and diversity of DFUs complicated by biofilm in vivo

Ten patients with chronic non-healing DFUs were enrolled. A total of 1306086 high-quality DNA sequences were generated (before = 623117, after = 682969), with a median of 61132 per sample-level data (range = 5702–168421). The OTUs identified 1976 unique taxa of which low-abundance OTUs were removed (<0.1%), leaving 124 OTUs for further analysis.

Confirmation of the presence or absence of biofilms in each DFU

Biofilms were visualized and confirmed in all 10 participants using SEM (Supplementary data Part S5). Biofilm architecture was graded using an arbitrary sliding scale from a score of 5 (heavy biofilm) to 0 (no biofilm) as previously reported.[17] The median value

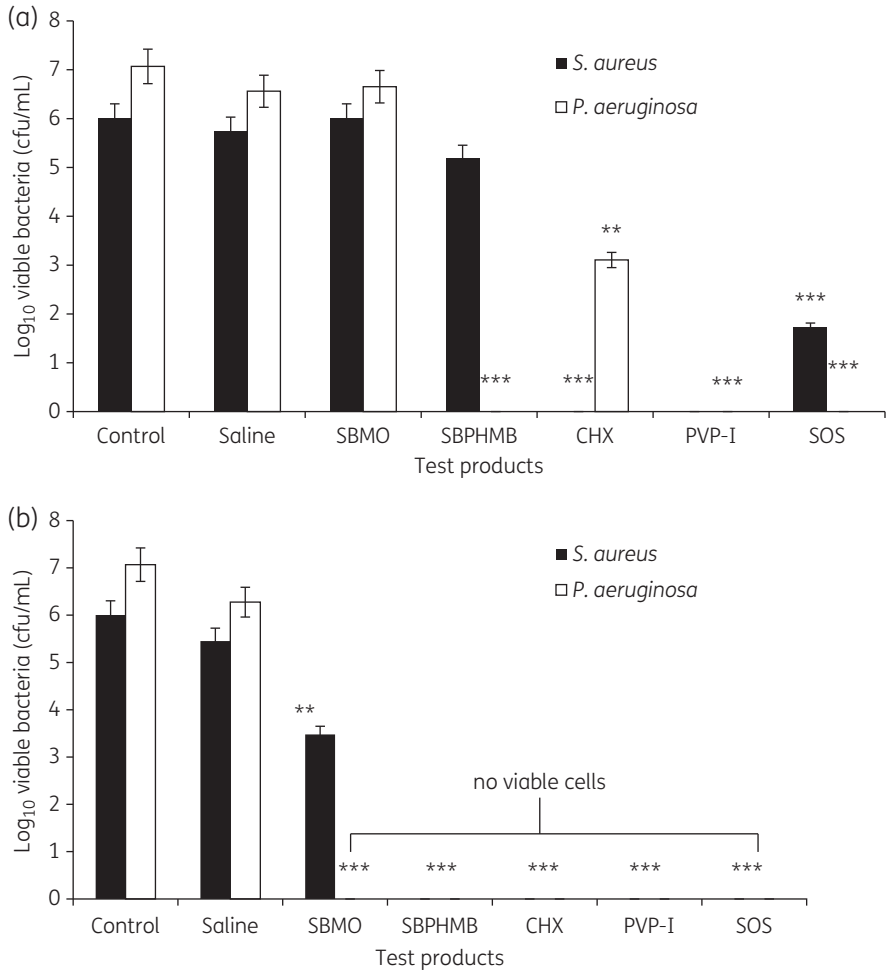

Figure 1. Effect of test products on bacterial viability. Bars represent means of logarithms of colony-forming units of viable biofilm cells after (a) 15 min exposure and (b) after 24 h exposure. Error bars represent the standard error of the means from three coupons (**$P < 0.01$, ***$P < 0.001$, no viable cells).

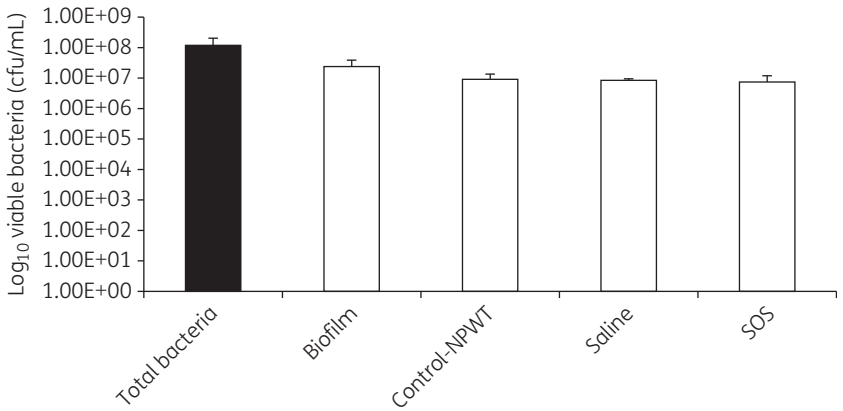

Figure 2. Treatment of porcine skin explants. 10^8 cfu of *P. aeruginosa* PA01 was inoculated onto porcine skin explants and after 3 days of growth at 37 °C, the average cfu of viable total bacteria or biofilm bacteria present before or after 12 cycles of 10 min instillations with saline or SOS solutions or only NPWT without instillation are shown. NPWT, negative pressure wound therapy.

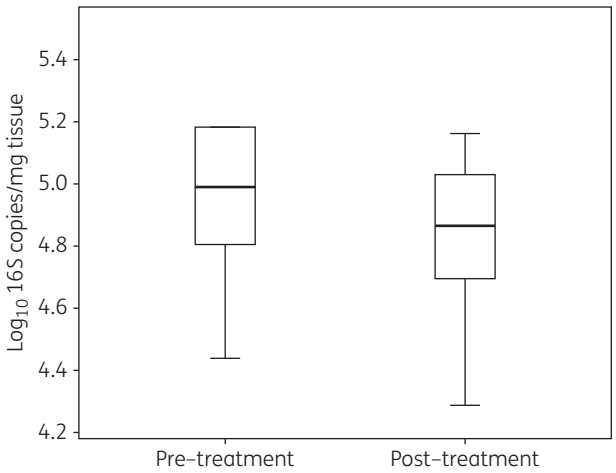

Figure 3. Effects of SBMO pre- and post-treatment of 10 chronic non-healing diabetic foot ulcers. Box-and-whisker plots show the median $\log_{10}$ 16S copies/mg of tissue values for all 10 patients.

of DFU biofilm architecture reduced from 4 pre-treatment (large microcolonies ~100 cells, and a continuous film/matrix) to 3 post-treatment (large microcolonies ~100 cells).

Microbial load of chronic non-healing DFUs complicated by biofilm

The application of SBMO for 15 min exposure daily, for 7 days, resulted in no change to the total microbial load (Figure 3) (median $\log_{10}$ microbial load pre-treatment = 4.9 $\log_{10}$ 16S copies/mg of tissue, versus 4.8 $\log_{10}$ 16S copies/mg of tissue, $P = 0.43$).

Analysis of community richness and diversity of chronic non-healing DFUs treated with SBMO

The most abundant OTUs contributing to >1% of the microorganisms within individual DFUs are shown in Figure 4(a); *P. aeruginosa*, *S. aureus*, *Anaerococcus* spp., *Prevotella* spp. and *Streptococcus* spp. were most commonly identified. The relative abundance of *P. aeruginosa* and *S. aureus* increased in all but one patient post-treatment with SBMO (Figure 4b), with pooled data from all samples identifying this to be statistically significant for the amount of *Staphylococcus* spp. DNA copies ($P = 0.04$). Only patient 9 seemed to experience a reduction in *S. aureus* levels (Supplementary data Part S6); however, a significant increase in *P. aeruginosa* was noted as a result (Figure 4a). Overall, there were increases in both aerobes and facultative microorganisms but these were reflected by a composite reduction in the relative abundance of anaerobic microorganisms (Figure 4d).

Microorganisms contributing to >1% of microbial communities in individual DFUs and from pooled data were analysed by alpha and beta diversity measures. Chronic DFUs prior to treatment were rich and diverse, yet there were minimal changes to community richness ($P = 0.3$), diversity ($P = 0.1$) or community composition of DFUs post-treatment ($P = 0.9$) (Figure 5a–c).

Discussion

Summary of key findings

We systematically tested the performance of topical antimicrobial solutions using short exposure times for *in vitro* and *ex vivo* models and an *in vivo* human trial. Our results suggest that the performance of these solutions is poor when challenged against mature biofilms using short exposure times that mimic real clinical use (i.e. 15 min application). Clinicians using topical antimicrobials to cleanse chronic wounds as a single therapy under the assumption of removing biofilm may therefore experience poor clinical outcomes. Clinicians should consider multifaceted strategies that include sharp debridement as the gold standard.[16]

What this study adds to the available evidence and new recommendations

There are many facets to the management of chronic wounds, with a large focus on managing wounds colonized or infected with either planktonic or biofilm phenotype microorganisms. While there is a plethora of data pertaining to the effectiveness of topical antimicrobials *in vitro* against both planktonic and sessile microorganisms, here we identify the inherent limitations of *in vitro* methodologies that fail to consider clinically relevant biofilm models when testing topical antimicrobials for use in wound care.[9–11] In particular, *in vitro* models testing topical antimicrobial wound solutions have not considered the clinical applications of the products' intended use with regards to the time of exposure,[11] and outcomes are often reported after 24 h exposure times. This does not reflect the typical clinical pattern of usage of these products or the 'instructions for use' explained in product inserts. Nor does the use of immature biofilms (early forming biofilm 24 h old) that have a less organized structure, a more active metabolism and a less pronounced stress response truly depict the complex, mature and highly tolerant biofilms identified in many chronic wounds.[3,18]

This may explain why some of the topical antimicrobials tested at clinically relevant times in this study performed poorly. Our *in vitro* model utilized two clinically relevant bacteria, *P. aeruginosa* and *S. aureus*, which have been noted as causes of delayed wound healing and as pathogens of infection.[2,19] Testing the efficacy of solutions over a single 15 min exposure time *in vitro*, we identified great variability in test performances. In general, surfactant-based topical antimicrobials performed poorly (except for SBPHMB against *P. aeruginosa*) and were no more effective than normal saline (non-antimicrobial).

Traditional antiseptics such as CHX and PVP-I were highly effective, while new-generation solutions such as SOS were also highly efficacious. CHX is a cationic bisbiguanide with a broad-spectrum biocide that is active against both Gram-positive and -negative bacteria.[20] Its primary action is against the negatively charged bacterial cell wall, leading to increased cell permeability resulting in cell death.[20] The efficacy of CHX in reducing or eradicating single or multispecies biofilm has been demonstrated *in vitro*,[21–23] with the combination of cetrimide and CHX producing enhanced antimicrobial activity (and anti-biofilm activity). One explanation for the effectiveness of CHX *in vitro* in this study may be the cationic surfactant properties of cetrimide, which has demonstrated the capacity to decrease the mechanical stability of biofilm (in addition

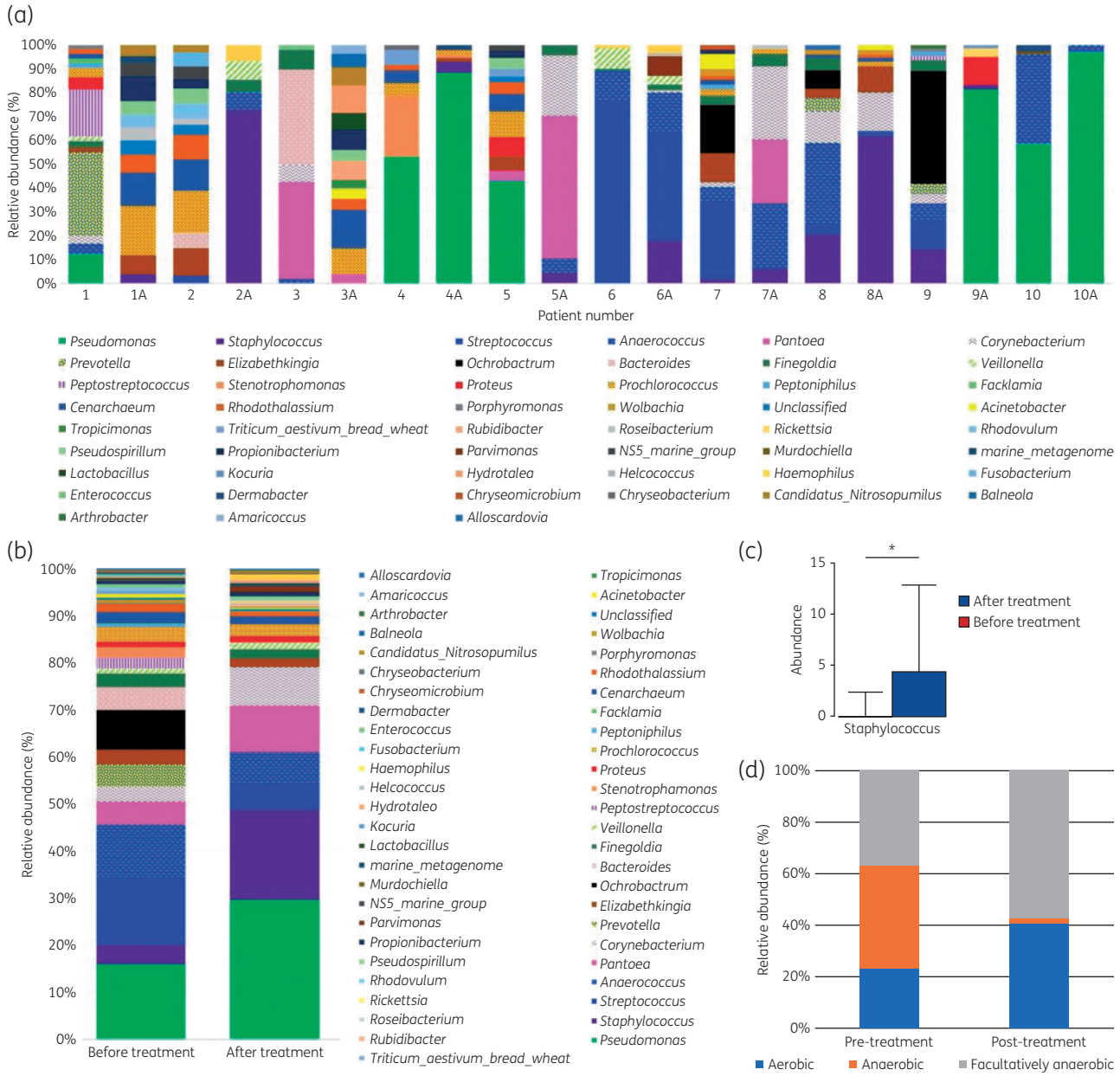

Figure 4. Effects on microbial communities following treatment with SBMO. Pairwise comparisons of pre- and post-treatment (A) microbial communities at the genus level in microorganisms contributing >1% within each wound. Further analysis of pooled data depicts changes across all ten patients when all DNA copies are pooled and examined. (a) Relative abundance (%) for individual wound-level data pre- and post-treatment. (b) Pooled data (all DNA copies) from ten patients identifies the relative abundance (%) of microorganisms pre- and post-treatment. (c) Relative abundance of pooled sample data of *Staphylococcus* spp. DNA copies pre- and post-treatment identifies a statistically significant increase ($P = 0.04$). (d) Relative abundance (%) of pooled sample data detailing the aerotolerance of microorganisms. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

to its proven bactericidal activity), but further work is required to elucidate further these effects in wound models.[24]

PVP-I also performed well *in vitro*, and as a broad-spectrum microbicide is capable of inactivating Gram-positive and -negative bacteria, bacterial spores, fungi, protozoa and several viruses.[25] PVP enables the delivery of free iodine to a target cell membrane, where it destabilizes the structural components of cell membranes.[25] It has demonstrated activity against biofilms *in vitro*;[26,27] moreover, more recent *in vitro* data on the performance of a wound care-related PVP-I on multispecies biofilms using the CDC reactor have corroborated the results of this study.[28]

More recently, 'newer generation' topical solutions with antimicrobial properties such as SOS have been utilized as anti-biofilm therapies in wound care, even in the presence of a low evidence base. SOS contains as a primary ingredient hyperchlorous acid (which is not new generation), and only one *in vitro* study is

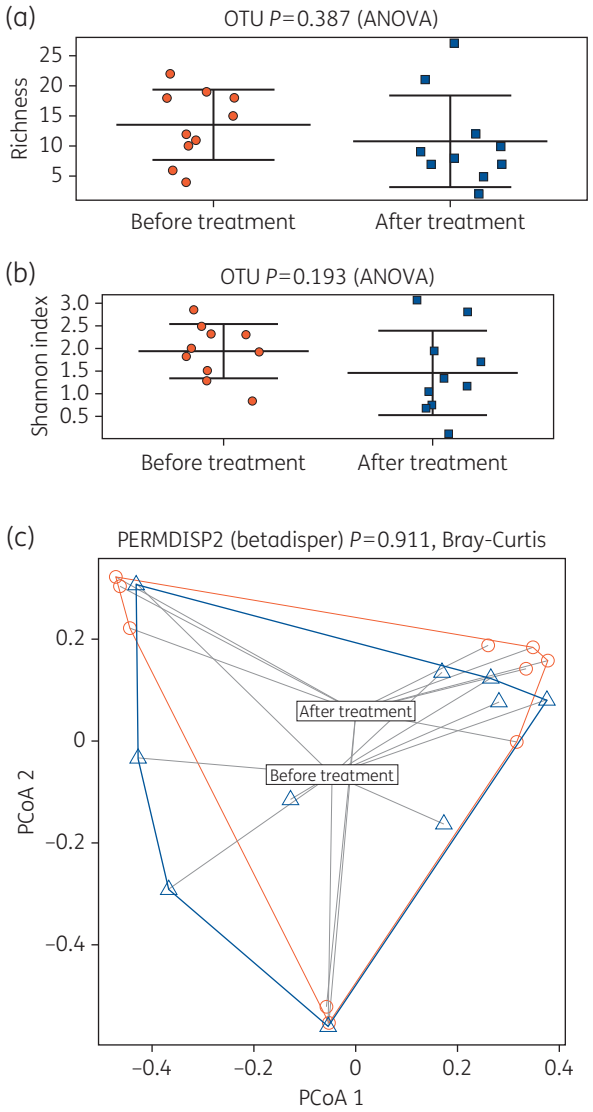

Figure 5. Alpha and beta diversity analysis pre- and post-treatment with SBMO. (a) The richness plot is a measure of the number of distinct or unique OTUs. These were reduced post-treatment but were non-significant. (b) The Shannon index is a measure of diversity that includes the number of unique microbial taxa and their relative evenness within each sample. Diversity of biofilm in diabetic foot ulcers post-treatment is reduced but non-significantly. (c) Principal coordinates analysis plots with Bray–Curtis distances between pre- and post-treatment samples identified that microbial communities are similar pre- and pre-treatment (blue triangles, pre-treatment; red circles, post-treatment). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

available that used the concentrations of SOS found in current wound care solution formulations.[10] Using a continuous flow tube reactor (to mimic the clinical scenario of a catheter) to grow mature 6-day-old *P. aeruginosa* PA01 biofilms, Sauer and colleagues[10] utilized SOS at the same concentration (80 ppm) reported in this study, to achieve a 2.5 $\log_{10}$ reduction after 60 min exposure.

Our study identified that SOS could eradicate *P. aeruginosa* biofilms in addition to performing well against *S. aureus* biofilm. This was in contrast to the porcine skin model, which identified that SOS achieved only 0.77 $\log_{10}$ reduction against *P. aeruginosa* PA01 biofilms. Potential explanations to describe these results could be the two different strains of *P. aeruginosa* that were used for the study. The *in vitro* model utilized *P. aeruginosa* (ATCC® 25619™) and the porcine skin explant utilized *P. aeruginosa* (PA01, ATCC® BAA-47™). Sauer and colleagues also utilized *P. aeruginosa* (PA01, ATCC® BAA-47™). Interestingly, the use of the *P. aeruginosa* PA01 strain yielded results that identified a reduced effectiveness of SOS. It is possible that whilst our *in vitro P. aeruginosa* (ATCC® 25619™) strain readily formed a biofilm with the characteristic *P. aeruginosa* architecture, it did not develop a high-level biofilm-specific resistance,[29] which may have arisen in the PA01 strain.

Other explanations for the different results observed for SOS *in vitro* versus the porcine skin model may be the surface the biofilms were formed on (i.e. the soft tissue dermal matrix of porcine skin, which more closely represented an actual wound bed compared with an abiotic polycarbonate disc). This may have contributed to alterations in microbial behaviour in response to the presence of biotic signals or organic material.[30,31] Biofilms grown on biotic substrates or *in vivo* often do not display the morphological or architectural characteristics of those grown *in vitro* (e.g. mushroom structures and towers), which are important parameters that undoubtedly affect bacterial behaviour.[32] Lastly unlike an abiotic surface, porcine skin has a striking similarity to human skin in terms of its structure and this is important given that microbial aggregates have been identified as not only forming on a wound surface, but also penetrating to deeper structures in a non-random distribution.[33] In this scenario, any topical solution applied to a contact surface would have to penetrate a biofilm formed on that contact surface in addition to then penetrating between tissue cells. This in itself presents a greater challenge (than that already posed by biofilm tolerance mechanisms) and may contribute to the reduced effectiveness of topical antimicrobials.

Lastly, the performance of SBMO was tested on human tissue in an *in vivo* study of chronic non-healing DFUs. SBMO was applied daily for 15 min over a 7 day treatment period, with the results identifying no change in the total microbial load from tissue biopsies. Interestingly, our *in vivo* results identified a correlation between the poor performance of SBMO against *P. aeruginosa* and *S. aureus* that was also seen *in vitro*.

Next generation DNA sequencing was performed to understand the effects of SBMO on microbial communities in chronic non-healing DFUs. The relative abundances of both *P. aeruginosa* and *S. aureus* within the majority of DFUs increased post-treatment. Conversely, an overall reduction in the relative abundance of anaerobic microorganisms and low frequency taxa (microorganisms contributing <1% relative abundance) was noted; however, the total microbial loads within these wounds did not decrease. This potentially suggests that more dominant species such as *Staphylococcus* spp., or *Pseudomonas* spp., benefit from the increased nutrient availability caused by disruption to the microbial community (that resulted from removal of competing microorganisms),[34] thus sustaining the microbial load within tissues.

Treatment with SBMO resulted in a reduction in the relative abundance of anaerobic microorganisms. Anaerobic microorganisms have been identified as part of polymicrobial communities cited for their involvement in delayed wound healing,[35,36] as pathogens of infection in the diabetic foot[37] and in biofilm production.[38]

In this instance reducing their numbers would seem like a positive step to reducing microorganisms with the potential to negatively impact the wound environment. Unfortunately, it is likely not this simple, particularly given the concomitant increases in pyogenic cocci (*Staphylococcus* spp.) and Gram-negative rods (*P. aeruginosa*), which are equal (if not greater) pathogens of infection.

To assess the overall effects of SBMO treatment on DFU microbiota (community richness, diversity, structure and composition) DNA sequence data were analysed using QIIME.[39]

Minimal reductions were seen in the number of OTUs (richness) and community diversity of chronic DFUs post-treatment. In a recent study by Loesche *et al.*,[40] the temporal analysis of chronic DFUs found that patient samples that received systemic antimicrobial therapy had no alterations to species richness or diversity, and that antimicrobial exposure did not drive microbiota variation. Instead the data indicated that antimicrobial exposure disrupted the microbiota where antimicrobials were specifically directed to treat underlying wound infection. We found a similar pattern of events with our data, in that exposure to SBMO had some effects when we explored our samples individually. For example, sample 2 experienced a significant disruption to its microbiota whereby pre-treatment *Staphylococcus* spp. contributed $<1\%$ relative abundance; post-treatment this significantly increased to $>65\%$. Similar patterns are seen across our data but it is not possible to infer if these changes would result in positive or negative effects to a wound. This intriguing aspect requires further correlation with longitudinal sampling that maps microbiota disruption to wound outcomes.

Our molecular-based data on the 16S gene, whilst informative in describing 'who is there', is unable to truly define 'who is doing what'.[41] In some wounds in which anaerobic microorganisms are acting synergistically with aerobic counterparts to increase pathogenicity or virulence in a chronic wound, their reduction may likely lead to positive effects. Conversely, and food for thought, any perturbations to the complex microflora seen within chronic wounds may lead to microbial dysbiosis. Of particular significance is the reduction in microbial diversity, which may directly contribute to pathogen selection and persistence.[42] Longitudinal studies are required to determine whether the alterations to the microbial diversity of chronic non-infected wounds seen by using topical antimicrobials lead to future complications.

Limitations

The CDC biofilm reactor used *in vitro* was performed under flow allowing mature biofilms to form on the polycarbonate coupons; however, this abiotic surface does not reflect the complexity of human tissue and the absence of the host immune response. Secondly, most chronic wounds are contaminated with multiple species of bacteria[3] and this study utilized single-species biofilms *in vitro*. That aside, our model tested clinically relevant exposure times against clinically relevant microorganisms involved in both chronic and infected wound types in screening the performance of topical antimicrobial solutions. qPCR was utilized to measure total microbial load *in vivo*;[15] however, this method has limitations in its inability to differentiate live or dead bacteria. The log reductions noted in this study therefore represent the minimal response and we acknowledge that some of the bacteria detected by qPCR could be dead, resulting in a lower calculable efficacy.

Overall, the limitations *in vitro* were circumvented by the addition of an *in vivo* study. Costs to perform this study were a limiting factor in not being able to test a wider range of topical antimicrobials *in vivo*. Further studies incorporating a human *in vivo* design may be required to understand the efficacy of single products tested in the *in vitro* stage of this study against microbial biofilms. However, when taking the group of studies performed collectively, there is a strong correlation between exposure time and efficacy.

Conclusions

Polymicrobial communities forming biofilms in chronic wounds may have extended time periods to develop complex, highly tolerant communities that differ greatly from single-species biofilm models grown on polycarbonate coupons for 24–72 h. The discrepancies between the three different test parameters in this study raise an important question about *in vitro* testing for anti-biofilm therapeutics, in which results identifying potential effectiveness against biofilm differ markedly when the test parameters are changed. *In vitro* testing for anti-biofilm strategies could be used as a screening tool for identifying potential therapeutics that may perform well at the next stage of testing (i.e. when taken to animal models or to clinical studies). The effectiveness of an anti-biofilm therapeutic at this *in vitro* stage is however not absolute, yet for many medical device companies this is the only data available for use in the promotion of products. This highlights the limitations of clinicians relying solely on *in vitro* data. When using porcine explants and human *in vivo* tissue samples, our data are highly suggestive that the exposure time of topical antimicrobial wound solutions and irrigation solutions is too short and that exposure time is critical in determining the efficacy of these products. Clinicians using these topical antimicrobial solutions as a sole therapy under the assumption of killing or eradicating biofilm should consider adopting multifaceted strategies that include sharp debridement as the gold standard.

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Transparency declarations

M. M. and G. S. have consulted for Smith & Nephew to undertake work not associated with this study but pertaining to producing educational material on biofilms in chronic wounds. All other authors have none to declare.

Supplementary data

Supplementary data Parts S1–S6 are available as Supplementary data at *JAC* Online.

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REVIEW

Approaches to biofilm-associated infections: the need for standardized and relevant biofilm methods for clinical applications

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ABSTRACT

Introduction: The concept of biofilms in human health and disease is now widely accepted as cause of chronic infection. Typically, biofilms show remarkable tolerance to many forms of treatments and the host immune response. This has led to vast increase in research to identify new (and sometimes old) anti-biofilm strategies that demonstrate effectiveness against these tolerant phenotypes.

Areas covered: Unfortunately, a standardized methodological approach of biofilm models has not been adopted leading to a large disparity between testing conditions. This has made it almost impossible to compare data across multiple laboratories, leaving large gaps in the evidence. Furthermore, many biofilm models testing anti-biofilm strategies aimed at the medical arena have not considered the matter of relevance to an intended application. This may explain why some *in vitro* models based on methodological designs that do not consider relevance to an intended application fail when applied *in vivo* at the clinical level.

Expert commentary: This review will explore the issues that need to be considered in developing performance standards for anti-biofilm therapeutics and provide a rationale for the need to standardize models/methods that are clinically relevant. We also provide some rational as to why no standards currently exist.

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1. Introduction

Since the early 1970s, an explosion of research on the concept of biofilms and their involvement in human health and disease has appeared in the medical literature [1]. This new wealth of information, driven largely by advancements in emerging technologies and techniques applicable to the study of bacterial populations *in situ*, have advanced the understanding of 'microbial biofilms.' The concept of biofilms in human health and disease is now universally accepted in chronic wounds [2,3] periodontal disease and dental caries [4,5], cystic fibrosis [6–8], in-dwelling medical device infection [9,10], otitis media and other upper respiratory infections [11,12], orthopedic infections [13], and tuberculosis [14].

Current definitions have described biofilms as microbes attached to surfaces or to each other in aggregates or clumps. They encapsulate in a self-produced extracellular polymeric substance (EPS) or matrix that can also contain host-derived components. As such, biofilms show extreme tolerance to antimicrobials and host defenses [15–18]. A plethora of *in vitro* biofilm models has elucidated that bacterial biofilms are more tolerant to antiseptics and disinfectants [19] as well as withstanding antimicrobial concentrations 100–1000 times higher than that of planktonic counterparts [20–23]. In spite of the wealth of research undertaken to identify biofilm tolerance to antimicrobials, no single causative mechanism has been identified. Instead, it has been suggested that a likely combination of factors contributes to biofilm tolerance [24,25] with several areas of interest including but not limited to; slow or incomplete permeation of antimicrobials through EPS [20,26], altered microenvironment and niches within biofilms promoting slow growth rates and adaptive stress response [27,28], efflux pumps [29], and the role of low frequency dormant 'persister' cells [30].

Regardless of whether researchers fully uncover the answers to the biofilm riddle of tolerance, the practical implications are that individual patients suffer with prolonged chronic infections that often require multiple rounds of antibiotics [31]. The current treatment strategy for chronic infections comes at a high cost to the healthcare system and, more importantly, to the patient, both economically and in the potential loss in their quality of life.

2. Exploring the concept of what is a relevant reduction for medically relevant biofilms?

Antimicrobial therapies for acute infections based on minimum inhibitory concentrations (MIC) (planktonic microorganisms' susceptibility to antibiotics) target rapidly multiplying

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planktonic microorganisms with high efficacy. Therapies based on MIC results employed against biofilm phenotype microorganisms that differ markedly in both their physiology and activity, typically fail to eradicate the problem, leading to a chronic infection for the patient. For some patients with indwelling medical devices the infection cannot be resolved until the material is completely removed [32].

Further clarity is required in understanding if this lack of correlation between conventional susceptibility test results, and therapeutic success in chronic infections may be reflective of biofilm presence. A recent Cochrane review on standard versus biofilm antimicrobial susceptibility testing to guide antibiotic therapy in cystic fibrosis identified that biofilm susceptibility testing was not superior to conventional antimicrobial susceptibility testing for biofilm [33]. In fact, the Cochrane review suggests that biofilm antimicrobial susceptibility testing may be more appropriate in the development of newer, more effective formulations of drugs that can be tested in clinical trials.

This aside, researchers have been driven to evaluate the efficacy of anti-biofilm strategies, using susceptibility test results based on assays that identify the minimum biofilm eradication concentration (MBEC) assay through *in vitro* models such as the Calgary biofilm device [34].

In addition to antibiotics, various alternate agents have also been explored for anti-biofilm strategies. These have included peptides, antiseptics, and oral and topical antimicrobials. How these agents are delivered to the biofilm have also varied greatly with mechanisms including coatings, drug eluting, wound gels, nanoparticles, irrigations, and solutions, all being explored. To further complicate the picture, several alternate techniques have been developed to quantify outcome measures of these agents *in vitro*. Biofilm biomass has been explored most typically in 96-well microtiter plates and flow systems using staining methods (crystal violet, Syto9 staining) with optical density (OD_{nm}) or confocal laser scanning microscopy (CLSM) to detect live/dead cells (expressed as percentages or OD_{nm}) [35,36]. Plate counts to enumerate viable cells that calculate antimicrobial efficacy expressed as cfu/ml, cfu/surface area, cfu/mg tissue have also been utilized.

Adding to the conundrum is the absence of a 'target' reference value required to ascertain the 'effectiveness' of anti-biofilm strategies in aiding the host immune response to clear infective microorganisms is profound. This has important consequences at a treatment level where clinicians often seek guidance from laboratory-based studies (often due the lack of *in vivo* data) in directing them to choose the most relevant and effective agent to reduce microbial colonization/infection. Granted, these decisions have historically been based around managing infections using planktonic paradigms, further highlights the requirement for data on the efficacy of anti-biofilm strategies.

Importantly, when deciphering what may be a 'target reference,' there are two sides of the fence to consider when posing questions around the performance standards of an agent that cites claims on 'effectiveness' or 'efficacy.' First, there is a regulatory perspective that looks to determine a 'target reference' based on standardized approaches using statistical attributes in determining the repeatability standard deviation and type I and type II error associated with an agent [37,38]. Undertaking this enhances statistical confidence in the outcome that an agent is efficacious. Second, is how well *in vitro* or *ex vivo* results translate to clinical efficacy and if those target references correlate to improvements in clinical symptoms and resolution of chronic infections.

With respect to the consideration of what would be a potential target value, no suggestions in the literature have been cited, and there are no data to support what a reasonable figure would be. This question in itself is complex given that a target reference value may move depending on the type of infection, the infecting strain, or the immune status of the patient. For example, data to support a reasonable target reference value for *in vitro* testing, must take into consideration that any changes in infectivity when bacteria are expressing biofilm phenotype *in vivo* may alter drastically. Until a clear path exists, then the most conservative approach is that the drug or device must demonstrate complete eradication of the biofilm in *in vitro* testing. The obvious approach to determine a reference value would be to transition from *in vitro* testing to *in vivo* clinical trials, as this would allow direct observations of what worked and what did not. In many cases, however, it may not be possible (an unethical) to obtain biofilm data directly from patients.

How the US Environmental Protection Agency (EPA) addressed this concern for human health biofilm disinfection claims is they are proposing a 6-log reduction in biofilm. The statistics tells us that if industry wants to be highly confident that they will achieve this target log reduction, then they need to formulate the biocide to completely kill the biofilm. What we are missing is data, and until that data is collected, the conservative approach would be to kill everything.

3. How should we approach assessing the 'effectiveness' of anti-biofilm therapies based on *in vitro* models in order to predict clinical results?

Throughout this article, the discussion of biofilm infections in patients has been purposefully over-simplified, given the complexity and breathes of discussing all concepts relating to how they contribute to human infection. Biofilms exist in many niches and vary significantly. This in itself, likely restricts the ability to develop an assay that closely mimics the exact architecture of an *in vivo* biofilm that could be used universally. Conversely, it is unlikely that an assay for every infection will be developed, given the large variation in biofilm architecture from *in vitro* to *in vivo*. Whether one is evaluating biocides for use against biofilm in toilets, or antibiotics to treat chronic wounds, it is virtually impossible to perfectly mimic an actual infection or environment in the laboratory.

A potential way forward for performance testing could be to develop a simplified biofilm assay that allows standardized adaptations (calibrated) to test parameters allowing the performance of a product to aid in predicting successful *in vivo* outcomes. Whilst no *in vitro* test will provide a direct answer to this, it does provide confidence to move forward with a very costly clinical trial.

Furthermore, it is not uncommon for researchers or testing laboratories to use standard methods beyond its original

intended use. For instance, a method designed and validated to test antimicrobial urinary catheters should not be used to study venous catheters without some significant modifications. This is similar to using a standard curve developed for chlorine to determine bromine concentrations – similar but not quite the same. Sometimes labs use the method because that is all they have available, this may be exemplified with 96-well assays or the CDC reactor. Once a laboratory starts using microtiter plates or CDC reactor, often they will just keep reapplying the same method to other applications. This raises questions how relevant the test is anymore. As researchers, we do our best to model what are thought to be the most important parameters to gain insights into how the biocide or antibiotic will perform when actually used. Ideally, there will be a menu of various methods designed for different application areas. This takes time and money, and right now researchers are in the foundation stages of model development.

Various publications have stated the need for the standardization of methods for assessing the 'effectiveness' of anti-biofilm therapies. A distinct problem, however, facing anyone attempting to decipher the literature and or attempting to replicate biofilm models for new therapies has been the lack of standardized methods for experimentally studying biofilms. This has caused much confusion when attempting to compare results between different research groups, and has led to large discrepancies when attempting to replicate the same results between different laboratories. The lack of methods also means there is no pathway for companies to follow when attempting to register a new device and/or drug with a regulatory agency.

In the most applied sense, standard methods development is the creation of laboratory protocols for the purpose of comparison, both within a single laboratory and among various laboratories. Researchers choose to use a standard method for various reasons. For instance, because every step of the laboratory process is exactly defined, a standard method is useful for teaching proper laboratory protocol or monitoring equipment performance. The impetus for the development of many microbial standard methods, though, is efficacy testing for product registration with a regulatory agency.

Regulatory agencies require efficacy data when a product is registered to ensure the quality, safety, and efficacy of antimicrobials (biocides, disinfectants, sterilants) in circulation (in a particular country). For this purpose, standardized methods that are repeatable, reproducible, rugged, and responsive are required [39]. A standard method should also be reasonable, meaning it should utilize equipment that is 'typical' for a laboratory and it should not require an excessive amount of time, supplies, or highly specialized training. Many biofilm research methods can uncover intriguing scientific insights even though the results are qualitative. However, regulatory authorities and standard setting organizations mostly prefer quantitative measures of efficacy.

In this manner, uniform test conditions permit comparison of results between products and laboratories. For a disinfectant to make a bactericidal claim for example, efficacy against planktonic *P. aeruginosa, Proteus vulgaris, E. coli,* and *S. aureus* is required by the Australian Therapeutic Goods Administration (TGO 54) [40], with similar organisms being required by other regulatory organizations. All these organisms are good biofilm producers and are associated with clinically relevant biofilm infections so it seems reasonable to include anti-biofilm testing for these (or a subset of these) organisms. Unfortunately, standard methods only exist for biofilms formed by *P. aeruginosa* (ASTM Methods E2196, E2562, E2647, and E2799).

Importantly, a relevant laboratory method should adequately emulate 'real use' conditions so that a laboratory test is predictive of how well a device (or test product of interest) will perform *in vivo*. Highlighting the decontamination of equipment and clinical surfaces with disinfectants/sterilants as an example, it is of importance for users (or clinicians) responsible for the decontamination of instruments or surfaces, to understand that the product they are using has been tested under conditions that best resemble the purpose they are to be applied, such as the hospital environment. Therefore, in terms of relevance, there are two basic strategies that researchers should strive to answer and that clinicians should strive to understand.

The first strategy is to engineer a biofilm in a laboratory test to have specific characteristics that emulates the biofilm *in vivo*, matching for example, the architecture, thickness, strength of attachment, and host factors such as proteins or immune cells. This is because alterations in any of these parameters can lead to alterations in the test outcomes, for example the sensitivity of biofilm to disinfectants varies with both the age of the biofilm and the method of growth [41]. This was well demonstrated in a paper by Buckingham-Meyer where kill rate (log reduction) decreased as the amount of shear on the test biofilm during growth increased.

The ASTM standard biofilm methods were developed based upon this relevance strategy (Table 1). By employing basic fluid dynamic concepts with regard to fluid shear and flow dynamics, the ASTM methods describe how to grow a biofilm that represents a general biofilm grown under high shear in a continuous stirred tank reactor (CSTR) (ASTM Method E2562) [42], in medium shear in a CSTR (ASTM Method E2196) [43], low shear in a plug flow reactor close to the air liquid interface (ASTM Method E2647) [44], and minimal shear in a batch reactor (ASTM Method E 2799) [45]. Others have recently reviewed the applicability of the biofilm reactors described in the ASTM methods for various applications [46–48].

The second basic strategy in methods development involves using reactors that incorporate the most important physiochemical and biological characteristics in the environment of interest [54,55]. An effective strategy that was followed for the development of the ASTM biofilm methods was to partition methods into sets of components. For testing the efficacy of disinfectants or antibiotics, these components include: growing a repeatable and relevant biofilm, applying the antimicrobial treatment, harvesting a sample of the treated biofilm, and analyzing the sample for viable cells. To better visualize this concept, Figure 1 shows a product testing and development guidance tree that outlines some of the numerous parameters under consideration for medically relevant biofilm standard methods.

In spite of the above, many researchers involved in biocide disinfection of a surface (not for medical devices or antimicrobial

Table 1. Commonly employed laboratory models for biofilm investigation.

Biofilm model	Method	Nutrient availability	Potential applications and relevance
Rotating disc reactor (annular reactor) (ATSM E2196 – approved 2002)	This test method is used for growing a reproducible *P. aeruginosa* biofilm in a continuously stirred tank reactor (CSTR) under medium shear conditions	Open system Dynamic Continuous flow	Rotating disc reactors are designed for laboratory evaluations of biocide efficacy, biofilm removal, and performance of anti-fouling materials. Example is to model a toilet bowl [49]. It is important to note that the rotating disk and CDC reactor were not originally designed to study medically relevant biofilms
Drip flow reactor (ATSM E2647 – approved 2008)	This test method is to grow, sample, and analyze a *P. aeruginosa* biofilm under low fluid shear and close to the air/liquid interface	Open system Dynamic Batch or continuous flow	DFR are employed for growing biofilms for direct *in situ* visualization. The DFR can model environments such as food-processing conveyor belts, catheters, and the oral cavity [50,51]
CDC biofilm reactor (ATSM E2562 – approved 2007)	This test method is used for growing *P. aeruginosa* biofilm under moderate-to-high shear. The resulting biofilm is representative of generalized situations where biofilm exists under high shear rather than being representative of one particular environment	Open system Dynamic Batch or continuous flow	Studies that utilized this reactor showed that it could be used for detecting biofilm formation, characterizing biofilm structure [52], and assessing the effect of antimicrobial agents on the biofilm (Note there is a large body of literature on how researchers are using the CDC, DFR and MBEC for various research applications)
MBEC assay /microtiter plates (ASTM E2799 – approved 2011)	This test method specifies the operational parameters required to grow and treat a P. biofilm in a high-throughput screening assay	Closed system Low shear (the reactor sits on a shaker) Batch	MBEC assay allows rapid throughput of multiple samples of anti-biofilm therapeutics such as antibiotics, antiseptics, compounds, and peptides [53]
Single tube disinfection (ATSM 2871 – approved 2013)	Standard test method for evaluating disinfectant efficacy against *P. aeruginosa* biofilm grown in the CDC biofilm reactor using the single tube method	The single tube method is only an efficacy test. Biocides are tested in a batch system, with no mixing at room temperature	This test was originally designed to determine the efficacy of liquid biocides against biofilm (bleach, quats, hydrogen peroxide blends, etc.). Although it has been optimized using biofilm grown in the CDC reactor, the original intent was that the biofilm could originate from any biofilm reactor, as long as the appropriate controls were carried along

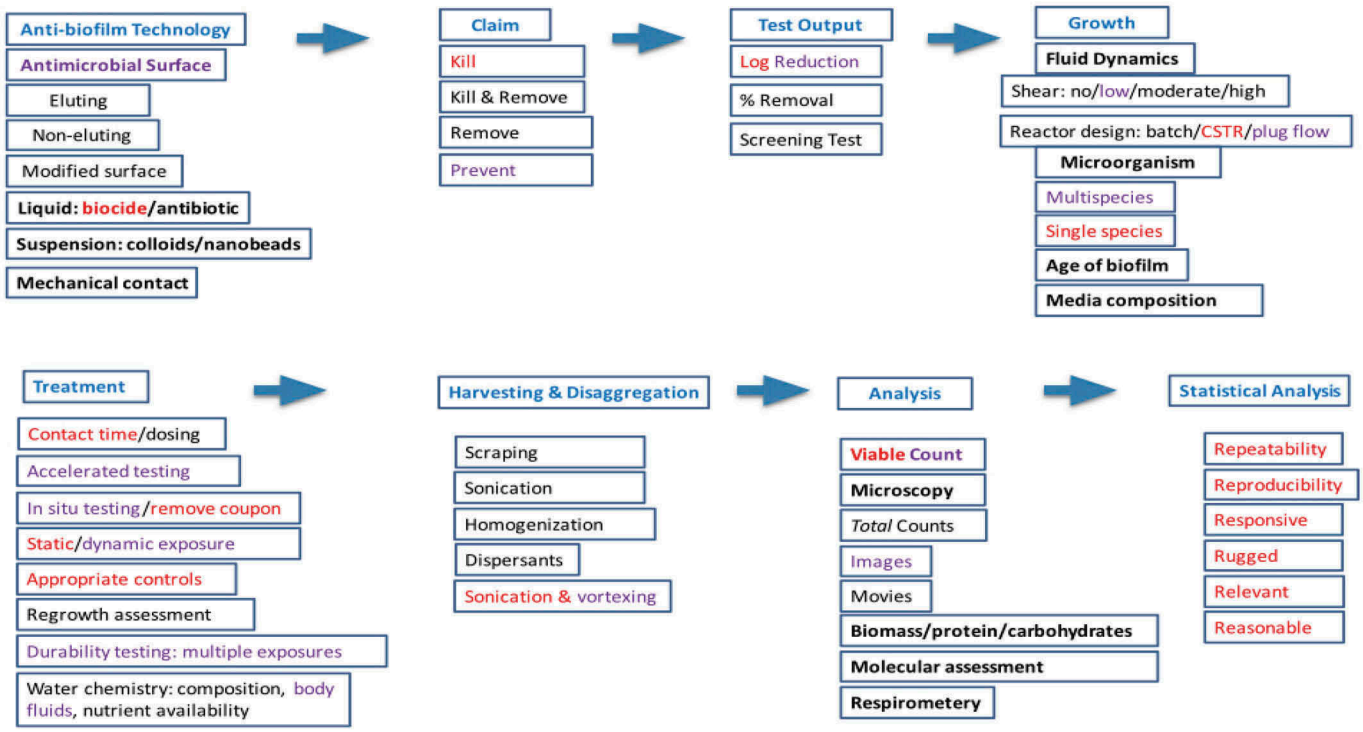

Figure 1. Product testing and development guidance. The decision making process begins with understanding the mechanism of action (MOA) of the of the anti-biofilm technology. The technology then determines the regulatory claim. For instance, an antimicrobial surface would most likely be associated with a 'prevents initial attachment' or 'reduces biofilm accumulation' claim, whereas a biocide manufacturer would most likely pursue a 'kills' or 'removes' biofilm claim. The claim then determines the necessary test output that will provide the necessary data to support the claim. For instance, a test that measures the log reduction in viable biofilm bacteria would provide the relevant data for 'kill' claim. The next step is to determine which laboratory growth and treatment methods best mimic the real world application. Various parameters of particular concern for biofilm methods are included in this figure, but it is important to note that the list is not exhaustive. The growth and treatment will often determine how the laboratory biofilm will be harvested and analysed. For instance, biofilm grown in microtitier plates is often not harvested, but stained directly and placed into a plate reader. Finally, every standard method must meet the statistical attributes listed in the figure. The text highlighted in red demonstrates the standardization path taken to measure the efficacy (kill) of biocides against biofilm. In this case, a single species biofilm is grown under high shear in the CDC reactor. The mature biofilm is removed from the reactor and tested under static conditions for a contact time specified by the biocide manufacture. Appropriate controls are always included. Sonication and vortexing is used to harvest the biofilm and the viable cells are enumerated using viable cells counts. Finally, the proposed method has undergone a collaborative study to verify that it meets the required statistical attributes. The text highlighted in purple demonstrates a potential strategy for testing antimicrobial surfaces engineered to prevent biofilm attachment. Full color available online.

therapies) may still pose questions such as how well does the hydrated biofilm formed on a coupon or in the MBEC plate represent biofilm on a clinical surface which is in a semi-dehydrated state and encased in thickened EPS? It is also unlikely that a biofilm formed on coupons or in an MBEC device will present the same challenge to biocides as biofilm that has been subjected to multiple rounds of decontamination, e.g. biofilm contaminating endoscope channels [56]. Biofilms form on all material types within the clinical environment, ranging from fabrics to plastics to stainless steel. Therefore, should research design questions be directed toward testing on different types of surfaces? For example, how relevant is a hard surface test as seen with current standards to killing biofilm on fabric? The CDC biofilm reactor (used in ASTM E2562) [42] uses removable coupons and thus has the capacity to compare different hard surface carriers with a range in free energy values and hydrophobicity, e.g. glass, plastic, porcelain, and steel. The premise of pushing the boundaries of any test condition and allowing researchers the 'artistic' flexibility to mimic 'real-use' conditions often increases the test methodologies in complexity. Typically, methods that try to exactly match every parameter of interest in this manner are complex and, therefore, when the method is verified in an inter-laboratory study (or ring trial), they do not perform well.

4. Expert commentary

Biofilm research as a whole has grown exponentially over the last two decades, yet there is minimal data correlating *in vitro* results to clinical outcomes. In addition, whilst the medical community has a greater awareness of the role of biofilms in human health and disease, there are still many areas of confusion for clinicians, who in particular find it difficult to understand how *in vitro* methods translate to something of clinical relevance [57]. This begs the question, why are we not further along in the battle against biofilm-associated infections? What is holding us back?

In trying to understand why the pursuit of new anti-biofilm therapies has been lethargic in some areas of medically relevant biofilm research, potential explanations are: (1) A lack of standardized methods for testing anti-biofilm models that is clinically applicable (to be discussed in the next section)

[58,59]. (2) The lack of regulatory guidance for setting performance standards for biofilm-related product claims in the medical device arena. (3) A poor understanding of what defines 'effectiveness' when applied to anti-biofilm strategies. (4) The slow response of industry in pursuing new anti-biofilm therapeutics, perhaps due to the lack of regulatory guidance, standard methods, the cost of research and development, and the cost of appropriate human clinical trials. These factors inadvertently force the industry to test their potential anti-biofilm therapies using methods that do not correlate to clinical outcomes. (5) Lack of funding resources to support the development of standard methods. (6) The slow progression in translating anti-biofilm research and therapeutics to clinically relevant information [47].

With the explosion in evidence detailing most aspects of biofilm involvement in human health and disease, clinicians and regulatory agencies have been hesitant to accept and pursue anti-biofilm treatment strategies. In contrast, the chemical disinfection world for example, has lobbied hard for anti-biofilm claims on products and these efforts have led to the development, validation, and approval of standard methods for testing of anti-biofilm products. Examples of these are five ASTM standard test methods (E2196, E2562, E2647, E2799, and E2871) (Table 1). The culmination of working toward developing standardized approaches that industry can utilize has meant that within the next 2 years, we may well see products with 'kills biofilm' claims.

However, the overall lack of advancement in anti-biofilms strategies from industry that include medical device/biocide companies maybe explained in their haste to scramble toward testing their current therapeutics. Historically promoted for use against planktonic microorganisms in acute infections, the drive to ascertain if they now have an action against biofilms may explain why industry are not diversifying away from traditional antimicrobials that have a high efficacy against planktonic microorganisms, and move toward new research and development specifically targeting anti-biofilm strategies. A major contributor for this is most likely the significant investment costs required to develop new therapies utilizing evidence from *in vitro* through to *in vivo*. The experimental designs in human studies for example would likely need to include a large number of patients for a statistically relevant conclusion to be reached. An example of this could be the bioengineering approaches to medical devices such as catheters and the lengthy processes required to bring a new device to market.

In tandem with a lack of investment from industry is the ever-increasing challenge to find funding to support the development of standard methods. The other point to consider is the time it takes to develop a standard method. Once a standard operating procedure is written, the method needs to go through rigorous intra-laboratory testing to accumulate sufficient data that supports that the method is repeatable, responsive, and rugged. This process may take 1–2 years, depending upon how compatible for standardization the research method is. The method is then taken to a standard setting organization where each step is critically reviewed and discussed, which may also take a few years. Finally, the method goes through a multi-lab collaborative study to determine the reproducibility of the method. Assuming the method performs well, the process is complete. But, if the method does not do well, it goes back to a standard setting organization (i.e. ASTM group) and is modified, extending the cost and time associated with standardizing it.

What is startling is why clinicians have not demanded the same development of anti-biofilm therapeutics? Or why medical device companies have been slow to pursue new therapeutics. One reason to explain this slow progress is when clinicians come across a new drug and/or device, the regulations on the wording of the claim/documentation is focused on curing or preventing infection. Biofilm does not become part of the discussion. This may seem to be a case of semantics, but simply not having biofilm be part of the discussion means generally it is not included as part of the clinician's decision-making in terms of infection management. With regard to an appropriate outcome, clinicians would also need to understand what 'effectiveness' of a product meant, whether biofilm was reduced (if so, by how much?) or if a 100% kill was achieved. Importantly, any reductions or killing of a biofilm would need to be associated with a reduction of infective symptoms and improved patient outcome.

For a change to happen, clinicians need to start asking if the patient has a chronic biofilm or an acute infection. In orthopedic device-, catheter-, or cardiac valve-related infections, clinicians are fully aware of the presence of biofilms. In fact, treatment is directed at biofilms with well-documented anti-biofilm activity such as fluoroquinolones [60,61] or rifampin [62], often in combination with another antibiotic [63], since resistance to rifampin can occur with a single point mutation [64]. In surgery, more aggressive debridement is also being practiced as an anti-biofilm strategy, treating biofilm infections more like an aggressive cancer which can come back with devastating consequences unless completely eradicated. However, specific biofilm-targeted treatment options are limited and if the biofilm is not eradicated there is an increased probability of generating resistance, leading to further complications for treating the infection down the road. This translates into demand for new strategies/treatments to cure biofilm infections.

Aiding in the confusion and lack of association between biofilm and chronic infection are the huge disparity in diagnostics. There are no diagnostic tools or biomarkers to help identify when biofilm is the driver of infection [65,66]. In the age of science-based medicine, how can clinicians be expected to deviate from standard measures of treating planktonic infections based on antimicrobial stewardships and make decisions to treat the infection as a biofilm infection, if there is no way to verify it?

When medical devices companies decide to pursue anti-biofilm strategies, they are faced with the barriers of navigating the minefield of regulatory standards. In this instance, regulatory agencies want clinical data that demonstrate a new drug or device's ability to decrease infection rates in patients. Historically, the regulatory tests to make these claims have been based on the MIC for planktonic microorganisms (Clinical laboratory standards institute (CLSI), M02-A12,

M07-A10, M100-S26). This is different than showing that a device prevents and/or reduces biofilm. Although logically, a person cannot develop a biofilm-based infection if no biofilm forms, but this is not the outcome that is being regulated or monitored by clinicians.

Even though researchers have demonstrated that biofilm is the root cause of many chronic infections, there is limited clinical biofilm data because clinicians do not routinely collect samples for biofilm-specific diagnostics. Granted this would be extremely challenging, but with advancements in new non-invasive technologies, the possibility certainly exists that a mechanism for collecting these samples will exist in the future. This can be exemplified in chronic non-healing wounds complicated by biofilm, where in general practice the clinician does not collect a swab or tissue sample of the wound bed to quantify the biofilm in order to direct antimicrobial therapy to treat the infection. Based upon data from industrial research, bacterial counts in the process water do not necessarily correlate to counts on the pipe's surface. This could also hold true in the human body. A low count in the urine does not mean that no biofilm is present; it just means that the biofilm has not grown to the point where the body is showing signs of infection. And of course, it would be unethical to do a study where catheters are removed over time to record the biofilm that forms, and correlate this number to when the 'typical' person begins to show signs of an infection (which is what occurs in industrial models for biofilm testing).

However, it is only useful to develop biofilm-specific sampling if clinical microbiology has the tools for appropriate diagnostics. Currently, confocal microscopy is considered the most direct way of demonstrating biofilms in clinical specimens [67] but these methods are time consuming and require highly specialized training.

This leads to a very important question. We do not know what the necessary log reduction in biofilm bacteria is that will ultimately cure the infection. For testing measures pertaining to the performance standards of an antimicrobial against planktonic microorganisms, the necessary reduction in microorganism counts has been defined as a greater than 3 log reduction (if the reproducibility standard deviation is 1 $\log_{10}$ then the antimicrobial must achieve a greater than 4 $\log_{10}$ reduction) [68]. Without knowing what this reference value is for biofilm-based infections, a conservative approach may be for regulatory agencies to require that the antibiotic/device must kill everything.

If this were the case, what does this mean for antimicrobial therapies currently employed against biofilms that exhibit only partial reductions against biofilms *in vitro*? Using the concept of rifampin, the more commonly employed antimicrobial against biofilms, evidence has identified it does not entirely kill the bacterial biofilm in most *in vitro* models [62,69]. Does this account to regulatory agencies not validating its future use against biofilm-associated infections, and/or clinicians not using it for the same purpose? Presently, the simple answer is no, clinicians will still likely use this systemic antimicrobial, evidence or not, because right now that is their only option and clinicians are committed to trying to help the patient to the best of their ability. Currently, the FDA would also not register an antibiotic or new drug based solely upon *in vitro* data as they are limited and do not take into account a human immune response, nor can any statements be made about preventing or curing infection.

Ideally, the systemic antimicrobial is working in combination with the person's immune system to resolve the infection, but the presence of a biofilm can restrict this response [70]. This may explain why people with chronic biofilm infections require multiple doses (and combinations) of systemic antimicrobials over extended periods of time (sometimes even in the order of magnitude of months or years). This exemplifies the main underlying theme of this review paper, which is that current *in vitro* biofilm tests are inadequate, and therefore are not predicting how the antibiotic will perform clinically. This may explain why there is little correlation between in *in vitro* and clinical results. The requirement for better designed (more clinically relevant) *in vitro* biofilm tests that have gone through a standardization process may improve clinical outcomes and help direct clinicians to using antimicrobials with proven efficacy against biofilms. Once the standard methods are in place, then correlation to clinical data and ideally performance standards will follow.

5. Five-year view

Is there a clear path toward the direction of standardized approaches to biofilm strategies? Many examples outlined in this review article highlight the biofilm-specific issues that need to be addressed in order to help provide better guidance to clinicians managing biofilm-associated infections. When the performance of an anti-biofilm strategy relates to the clinical care of patients, there is a need to achieve a standardized biofilm methods 'utopia.' This will provide pharmaceutical/device manufacturers all the experimental parameters required so that a collaborative study may be done. From a regulatory perspective, this would also allow for the method's reproducibility standard deviation (SD) to be determined. This requirement is highly relevant for clinicians to appreciate, who may read a paper on a new technology that performed fabulously in a one laboratory study, did fine in an animal model, but failed miserably in a clinical trial. If an appropriate statistical analysis had been performed, the probability of failure would have been predicted. In general, a large percentage of experiments may lack the statistical attributes that are required of a standard method, and without statistics, there is no statistical confidence in the outcome.

In the same instance, there needs to be delineation between absolute standard methods and research methods, with the latter affording the flexibility for researchers to advance new therapeutic strategies toward biofilm-associated infections. Roberts and colleagues made reference to this notion, suggesting that researchers should not be afraid of undertaking initial *in vitro* screening (nonstandardized experiments) (Roberts et al. 2016).In doing so, this may provide greater predictive power for *in vivo* activity, and also allow side-by-side comparative studies with established antimicrobial agents. This may actually enhance the capability to better understand biofilm-associated infections.

However, Roberts and colleagues make the same conclusion as we would, which is the most relevant system should be

used based upon the questions being asked. Although preliminary experiments will allow researchers to make advances in our basic understanding of these biofilm infections, regulatory agencies require data collected with methods that have been statistically validated, which generally means the method has gone through a standardization process. Perhaps, it is the reluctance of medical researchers to use standard methods that has provided a roadblock and explains why the medical field lags behind the biocide/industrial field with regard to biocide claims.

Key issues

- Biofilms show remarkable tolerance to many forms of treatments and the host immune response.
- The lack of correlation between conventional susceptibility test results and therapeutic success in chronic infections maybe reflective of biofilm presence.
- The absence of a 'target' reference value required to ascertain the 'effectiveness' of anti-biofilm strategies to clear infective microorganisms suggests complete eradication is required.
- A potential way forward for performance testing could be to develop a simplified biofilm assay that allows standardized adaptations (calibrated) to test parameters allowing the performance of a product to aid in predicting successful *in vivo* outcomes.
- No *in vitro* test provides a prediction on how well a product will work *in vivo*, but it does provide confidence to move forward onto animal models or costly clinical *in vivo* trials.
- Many areas of confusion regarding anti-biofilm strategies still exist for clinicians who are caught either; 1. Finding it difficult to understand how *in vitro* methods translate to something of clinical relevance or 2. Think successful *in vitro* outcomes will provide similar results *in vivo*.

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