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Role of anaerobes in polymicrobial communities and biofilms complicating diabetic foot ulcers

Steven L Percival[1] | Matthew Malone[2,3] | Dieter Mayer[4] | Anne-Marie Salisbury[1] | Gregory Schultz[5]

[1]5D Health Protection Group Ltd, Centre of Excellence in Biofilm Science and Technologies (CEBST), Liverpool Bio-Innovation Hub, Liverpool, UK

[2]Infectious Disease and Microbiology, School of Medicine, Western Sydney University, Sydney, Australia

[3]High Risk Foot Service, Liverpool Hospital South Western Sydney LHD, Sydney, New South Wales, Australia

[4]Department of Surgery, HFR Fribourg – Cantonal Hospital, Fribourg, Switzerland

[5]Institute for Wound Research, Department Obstetrics & Gynaecology, University of Florida, Gainesville, Florida

Correspondence
SL Percival, 5D Health Protection Group Ltd, Centre of Excellence in Biofilm Science and Technologies (CEBST), Liverpool Bio-Innovation Hub, Liverpool, UK.
Email: steven.percival@5dhpg.com

Infected tissues in the feet of people with diabetes in the form of a diabetic foot ulcer (DFU) present a complex pathology for clinicians to manage. This is partly attributed to the multi-factorial nature of the disease, which may include; altered foot architecture leading to excessive plantar pressures and frictional forces peripheral arterial disease and loss of protective sensation. In addition, to the above co-morbid variables, it is understood that a delayed wound healing state may be perpetuated by the presence of microorganisms residing in the wound tissue. The microbiology of chronic DFUs has often been reported as being polymicrobial. Of growing interest is the presence and potential role of anaerobic microorganisms in the pathology of DFUs and how they may contribute to the infective process or delayed healing. The presence of anaerobes in DFUs has been greatly underestimated, largely due to the limitations of conventional culture methods in identifying them from samples. Advancements in molecular and microscopy techniques have extended our view of the wound microbiome in addition to observing the growth and behaviour (planktonic or biofilm) of microorganisms in situ. This review paper will reflect on the evidence for the role and significance of anaerobes in DFUs and infection. A focus of this review will be to explore recent advancements in molecular genomics and microscopy techniques in order to better assess the roles of anaerobic bacteria in chronic DFUs and in biofilm-based wound care.

KEYWORDS

anaerobes, biofilms, diabetic foot ulcers, wounds

1 | INTRODUCTION

The causality of a diabetic foot ulcer (DFU) is considered multi-factorial in origin. Such factors can include peripheral vascular disease, peripheral neuropathy and some form of trauma which may inflict damage to the skin. Once the skin is breached underlying structures of the skin and soft tissue are exposed to planktonic commensal microorganisms or opportunistic pathogens. DFUs present an ideal environment for harbouring microorganisms as they offer a warm, moist and nutritive home especially if devitalized tissue is present in the wound bed. When this is combined with several aspects of altered immunologic function,[1] it may explain why some bacterial infections in people with diabetes persist despite optimal care.

Infections in the feet of people with diabetes have been classified by both the International Working Group for the Diabetic Foot (IWGDF) and the Infectious Disease Society of America Diabetic Foot Infection guidelines (IDSA).[2,3] Both have promoted the diagnosis of infection be based on "clinical diagnosis" using greater than two clinical signs; inflammation erythema local tenderness or pain warmth and purulent discharge. Importantly, through traditional culture and microscopy approaches, the microorganisms responsible for acute infections have been identified as being planktonic; that is being single cells that rapidly multiply when in exponential growth and are susceptible to antibiotics if not inherently resistant.

Alternatively, a growing body of evidence has identified that many microorganisms may not exist in this planktonic

phenotype. Instead some microorganisms prefer to aggregate in communities of slow growing cells in a biofilm phenotype. Biofilms have been described as a coherent cluster of bacterial cells imbedded in a biopolymer matrix (EPS) which compared with planktonic cells shows increased tolerance to antimicrobials and resists the antimicrobial properties of the host defence.[4] EPS makes up the largest component, often over 80% of its volume and is composed of polysaccharides, proteins, metal ions and extracellular DNA (eDNA).[5]

Recently, Johani and colleagues[6] reported evidence confirming that biofilms are ubiquitous in both infected and non-healing DFUs. This evidence further supports previous work from other researchers using microscopy approaches that have visualised biofilm structures across varying wound etiologies.[7–10] Furthermore, a recent systematic review and meta-analysis of wound biofilms reported up to 78% of chronic non-healing wounds likely contained biofilm.[11] Taken collectively, the available data suggest that biofilms have the potential to affect many wounds. Of these problems, the presence of a biofilm is thought to delay wound healing[12] and contribute to chronic infections.[13]

This review will focus on the microbiology that contribute to DFUs in particular exploring old and new evidence to help clarify the significance and role of anaerobes that contribute as either planktonic microorganisms in acute infection or as members of polymicrobial biofilm communities.

1.1 | Bacteriology of DFUs; A view from conventional culture data

A large amount of data from authors utilising conventional culture methods have identified that the most infected DFUs are complicated by pyogenic cocci such as *Staphylococcus aureus* and beta-hemolytic Streptococci.[14,15] Most newly detected infected DFUs of shorter duration have also been reported as being complicated by mono-microbial organisms and conversely chronic infections seem to harbour more complex polymicrobial communities.[11]

Despite a plethora of studies conducted to investigate the predominant species of microorganisms present in DFUs the role of anaerobes in DFUs, as well as biofilms, has been largely under researched and thus potentially underestimated.

An early study by Ramani and colleagues[16] identified 162 anaerobe isolates and 61 aerobe isolates in 75 patients with DFUs. Within this study, *S. aureus* was the most common aerobic microorganism isolated from 60% of cases and *Bacteroides fragilis* was the most common anaerobic microorganism to be isolated. In a more recent study by Shanmugam and Jeya,[17] the bacterial profile and antibiotic resistance patterns of 50 patients with DFUs were analysed using traditional culture methods. The most predominant species identified included *Pseudomonas spp* (16%) *Escherichia coli* (14.6%) and methicillin-sensitive *Staphylococcus aureus* (13.3%). The other organisms that were isolated were *Streptococcus pyogenes* (10.6%) *Klebsiella spp* (8%), *Acinetobacter spp* (8%), methicillin-resistant *Staphylococcus aureus* (MRSA) (8%), *Proteus mirabilis* (6.6%), *Citrobacter spp* and *Enterococcus spp* (5.3%) each, CoNS (2.6%) and *Enterobacter spp* (1.3%).[17]

Key Messages

- the polymicrobial nature in DFUs is clearly evident but there is still a large gap in knowledge over the distinct effect of anaerobic bacteria in the chronicity of DFUs
- despite there being a small number of papers that identify the presence of biofilms within chronic wounds basic microbiological methods and even some molecular methods do not give us insight into the structure of a polymicrobial biofilm within these DFUs
- studying and investigating the physical and chemical characteristics of "true to life" biofilms still remains a significant challenge

An additional study conducted by Tiwari and colleagues[18] on 62 DFUs found *Escherichia coli* to be the most common isolated species followed by *Staphylococcus aureus.*[18] Microbiological specimens were obtained from tissue biopsies and cultured under aerobic conditions. It was also reported in this study that 21% of the samples were culture negative despite one of the subject ulcers was producing a foul smell and showing maggot infestation casting further doubt on the accuracy and usefulness of culture methods to identify pathogenic bacteria in clinically infected wounds.[18]

Similar microbiological results were obtained by Zubair and collegues[19] who identified *Staphylococcus aureus* as the most common species isolated from 62 patients with DFUs followed by *Escherichia coli* and *Pseudomonas aeruginosa.* The samples obtained during this study were only processed for aerobic bacteria with no reference to anaerobic bacteria.[19]

Pathare et al.[20] found that superficial ulcers and those of shorter duration were associated with higher relative abundance of Staphylococcui in their study of DFU's that used tissue cultures. However, Rouhipour and colleagues[21] studied 149 patients over a 1-year period isolating 546 opportunistic pathogens. Samples were analysed for both aerobic and anaerobic species. The most common opportunistic pathogens were found to be *Enterococcus spp* and methicillin-resistant *Staphylococcus aureus* followed by *Escherichia coli* and *Pseudomonas aeruginosa.* In addition, 1.7% of isolates were anaerobic bacteria the majority of which were *Peptostreptcoccus spp* followed by *Bacteroides fragilis.*

A study by Al Benwan et al.[22] of 440 patients with DFUs found 777 opportunistic pathogens, 15.3% of which were

anaerobes. The most common isolated microbes overall included those belonging to the *Enterobacteriaceae* group followed by *Pseudomonas aeruginosa* and *Staphylococcus aureus* and Gram-negative anaerobic organisms. Anaerobes were found to be present in 49% of patients in a study by Citron et al.[14] by which 454 samples were obtained from 433 patients presenting with clinically infected DFUs. The most predominant aerobic species isolated were *Staphylococcus spp* followed by *Streptococcus spp* and *Enterococcus spp* whilst the most prevalent anaerobic species included Gram-positive cocci; *Prevotella spp*, *Porphyromonas spp* and *Bacteroides fragilis.* The majority of anaerobic species were found alongside aerobic organisms with only 1.3% anaerobes found in single culture.[14]

1.2 | Advancements in molecular genomic approaches have detailed the microbiome of DFUs

Molecular techniques that include next generation DNA sequencing have extended the view of the microorganisms residing in wounds including DFUs. The vast majority of studies which have utilised these techniques have focused on amplifying and sequencing the 16S rRNA gene. This gene is a perfect option to target and amplify firstly because it is only present in bacteria thus making differentiation between microbial DNA and human DNA easy. Over the past decade data from microbial communities of infected and non-infected tissue have provided a greater insight into the microorganisms residing in ulcerated tissue.

One of the major insights highlighted from molecular microbiology has been the significant underestimation in the presence and potential involvement of anaerobic microorganisms. Malone et al.[23] recently published a review article on the application of molecular techniques for better understanding diabetic foot infections. The review provided an insight into how these techniques may change the future of microbiology in addition to reviewing the current evidence from authors employing these techniques for wound research. As such this review will briefly summarise these findings with a focus on anaerobes.

Rhoads et al.[24] investigated 168 chronic wounds by culture techniques rather than molecular methods (16S rRNA). Culture identified 17 different bacterial species in contrast to molecular methods that identified 338 bacteria species. Interestingly, out of the top 20 bacteria identified using molecular techniques 9 of the taxa were identified as anaerobes. Unfortunately, in this study culturing anaerobic bacteria was not undertaken.

Gardner and colleagues[25] conducted a study on 52 patients using high-throughput sequencing of the bacterial 16S rRNA gene and attempted to correlate microbial findings to clinical relevance. Ulcer depth was associated with the abundance of anaerobic bacteria and negatively correlated with abundance of Staphylococcus. Naturally ulcer duration was also found to positively correlate with bacterial diversity species richness and relative abundance of Proteobacteria and again negatively correlated with the presence of *Staphylococcus.*

Dowd and colleagues[26] (using a bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP) have evaluated the bacterial diversity of 40 chronic DFUs. The most prevalent bacterial genus associated with diabetic chronic wounds was *Corynebacterium* (30 of 40 DFUs average abundance = 14.4%) followed by anaerobic genera of *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%). Other minor bacterial communities also included common *Serratia, Staphylococcus and Enterococcus spp.* This study provides evidence to support the concept of functional equivalent pathogroups (FEP). As previously explained, individual members of these communities may not cause disease when they occur alone. However, when they co-aggregate into FEP the synergistic effect provides the biofilm community the factors necessary to maintain chronic biofilm infections.[26] Using this same molecular technique of bTEFAP pyrosequencing, Drake and colleagues (who evaluated the bacterial diversities of pressure ulcers) concluded that anaerobic bacteria even though in minor populations were present in all wounds suggesting that they may play a significant role in the aetiology of chronic wound infections.[27]

Dunyach-Remy et al.[28] conducted a pilot study to evaluate the contribution of polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) in the microbiological diagnosis of DFUs compared with conventional techniques. Using conventional culture methods pathogenic bacteria typical to what has been described previously were identified: *Staphylococcus aureus*, β-haemolytic *Streptococci, Enterobacteria* and anaerobes whereas commensal flora included; cutaneous bacteria (coagulase-negative *Staphylococci*, *Corynebacteria* and *Acinetobacter spp.*). Intermediate flora included low-virulence bacteria such as *Pseudomonas aeruginosa, Enterococcus spp* and *Stenotrophomonas maltophila.*

Taken collectively many studies using both culture-dependent methods and DNA/RNA molecular identification methods have identified that the vast majority of the isolates found in DFUs are pyogenic cocci such as *Staphylococcus aureus.* Conversely, many molecular studies also highlight that anaerobic microorganisms are under-represented. Importantly, these studies show that wound samples can harbour complex polymicrobial communities that include both aerobic facultative and anaerobic species all in the same wound. This brings into the question of how aerobic species can co-exist with anaerobes. One answer maybe the formation of biofilm.

1.3 | Biofilm: the microbial city allowing coexistence between aerobes and anaerobes?

Once planktonic microorganisms alter their behaviour (phenotype) through attachment and microcolony formation to

progress into mature biofilms the residing microorganisms, their interaction with one another and environmental pressures largely control the maturation process.[29] This social interaction between microorganisms is both an area that has divided researchers and spawned an entirely unique area of research termed "sociomicrobiology".[30] The importance of bacterial cooperation versus competition is highly relevant to the 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.[31]

In mixed species biofilms, bacterial aggregation and microcolonies do not seem to form by random chance. Instead biofilms can form communities with similar requirements and vested interests and settle in microenvironments where survival is possible through the power of cooperation.[31–33]

Early microelectrode studies of aerobic *in vitro* biofilm models found discrete areas within biofilm that had significant oxygen depletion.[34] 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.[35] Recent data by James et al.[36] has provided further evidence to support a concept of a localised low oxygen tensions contributing to wound chronicity. Using oxygen microsensors and transcriptomics (examining microbial metabolic activities) to study in situ biofilms, James and colleagues[36] identified steep oxygen gradients and induced oxygen-limitation stress responses from bacteria. James and colleagues identified through transcriptomics 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, these data support the concept of a biofilm establishing and maintaining localised low oxygen tensions in a wound.

1.4 | Anaerobes in multi-species wound biofilms

In order to appreciate the significance of anaerobes in DFUs it is necessary to address how biofilms are studied and modelled including how anaerobic bacteria interact in a biofilm environment. A key issue when discussing the significance of biofilms in chronic wound healing is the lack of appropriate laboratory models and human *in vivo* data available to investigate the underlying mechanisms involved. Although there is overwhelming evidence for the polymicrobial nature of biofilms, many models have been based on a single bacterial species, for example the *in vivo* model using rabbit ears created by Gurjala and colleagues uses *Staphylococcus aureus* as the single microbe.[37] This does not accurately represent the microbiota in most chronic wounds failing to account for interspecies interactions and possible synergistic effects from a polymicrobial environment. Although experimentally it is difficult to form polymicrobial biofilms a study by Sun et al. demonstrated polymicrobial biofilm formation with the following anaerobic species: *Peptoniphilus ivorii*, *Peptostreptococcus anaerobius*, *Anaerococcus lactolyticus*, *Finegoldia magna* and *Clostridium perfringens*).[38] The ability to model anaerobes in biofilms especially with multiple species present represents a huge advancement in chronic wound research.

Similarly, Dalton and colleagues have shown success when incorporating four key species commonly found in chronic wounds with the inclusion of an anaerobic species in an *in vivo* model.[39] After initial problems due to *Pseudomonas aeruginosa* over-populating the model when grown in a planktonic state, the researchers found that forming the biofilm *in vitro* then transplanting it into the murine model resulted in successful biofilm growth with contributions from all four bacterial species. Dapa and colleagues[40] focused research into anaerobic microorganisms in gut biofilms and showed a linked between spore formation and biofilm formation in a clinical strain of the anaerobe *Clostridium difficile*. Furthermore, *C. difficile* biofilms were more resistant to high concentrations of vancomycin.[40]

Many studies have investigated *in vitro* biofilms which although effective for investigating experimental aspects such as ease of use and repeatability are less representative of a clinical biofilm.[41] *in vivo* models allow the investigation of host responses against infection as well as determining the effect of the biofilm on physiological healing processes such as wound closure and skin barrier function. Typical models for *in vivo* investigations include small mammals[42] as well as porcine models. Whilst small mammals tend to have lower associated costs and can be kept and handled easily it is widely accepted that porcine skin has more similarities to human skin and, therefore, is preferential in wound healing models.[43]

There is little evidence to support the effect of anaerobic bacteria in cellular integrity in such chronic wounds as DFUs. Stephens and colleagues[44] showed that anaerobic cocci isolated from venous leg ulcers significantly inhibited keratinocyte and fibroblast proliferation at high concentrations and at low concentrations profoundly inhibited keratinocyte wound repopulation and endothelial tubule formation.[44]

Evidence from *in vivo* human DFUs implicating anaerobes as part of multi-species biofilm communities have recently been cited in the literature.[6,45] Malone et al.[45] utilised next generation DNA sequencing to explore the microbiome of 39 infected and chronic DFUs.[45] A further 65 DFU tissue samples subjected to scanning electron microscopy (SEM) and peptide nucleic acid fluorescent in situ hybridisation (PNA-FISH) to confirm the presence of both mono and multi-species biofilms.[6] Firstly, the microbiome of DFUs confirmed the usual suspects of *Staphylococcus aureus, Corynebacterium striatum* and *Streptoccocus agalactiae* were predominant. The data, however, also identified that anaerobes in particular

members belonging to *Clostridales family XI* (*Finegoldia spp*, *Peptoniphilus spp*, *Anaerococcus spp*) were extremely common in many wounds. In fact, almost every wound contained anaerobes with some wounds containing up to as much as 80% anaerobes of the total number of microorganisms recovered from the DFU. The analysis of the microbiome also identified that anaerobes often co-existed with aerobic microorganisms. This may largely be attributed to the fact that the DFUs sampled had biofilm as confirmed via the above techniques by Johani et al.[6] They utilised microscopy techniques in combination with molecular methods to show that DFUs are composed of both mono and polyspecies biofilms and some DFUs contain both in the same sections of tissue.

1.5 | Synergy between aerobic and anaerobic microorganisms

Due to the polymicrobial nature of DFU's it is important to consider microbial interactions in pathogenicity. An early study by Kelly[46] showed synergistic interactions between *E. coli* and the anaerobe *B. fragilis.* In an animal model of intra-abdominal sepsis coliforms were responsible for acute peritonitis septicaemia and shock while the anaerobes particularly *Bacteroides fragilis* caused the late complications of abscess formation.[47] Brook and colleagues[48] investigated mixed aerobic facultative and anaerobic cultures in the subcutaneous abscesses of mice and discovered that there was a significant increase in mortality when each aerobic microorganism was mixed with Bacteroides species and Gram-positive anaerobes were mixed with *P. aeruginosa* or *S. aureus.*

Synergistic interactions between microorganisms within a biofilm affect the biovolume and biofunctionality of the biofilm.[49] In the context of cystic fibrosis biofilms consisting of *P. aeruginosa* and either the emerging species *Inquilnus limosus* or *Dolosigranulum pigrum* increased the tolerance of the overall consortia to most antibiotics without a change in the number of biofilm-encased cells.[50]

1.6 | Does the presence of anaerobes require a change to clinical practice?

Dowd and colleagues[26] also agree that using typical culture methods failed to identify major contributing populations especially strict anaerobes within the given wound types of all chronic wounds. Furthermore, stating that these methods are inherently biased as they examine only the 1% of all microorganisms which are able to grow in pure culture.

Metronidazole is a typical drug of choice for anaerobic infection in DFU for a majority of clinicians. Aherrao and colleagues[51] conducted a study to determine if metronidazole is effective in the treatment of and healing of anaerobically infected DFU's. Deep tissue samples from the wound area of 61 diabetic foot patients were tested for anaerobic bacterial infection (*Peptostreptococcus productus, Bacteroides* and *Clostridium*) by PCR. PCR-positive patients were then randomised into 2 groups: metronidazole and non-metronidazole. Antibiotics for the control of the infection were given to both groups as per clinical condition of patients with treatment outcomes being assessed by the complete closure of the wound. Interestingly, out of 61 patients, PCR detected evidence of anaerobic infection in 52% of the patients; direct comparison culture methods only detected an 8% infection hence emphasising the significance of the PCR technique over culture methods in the detection of microbes. In this study, *Clostridium* was found with highest prevalence followed by *Bacteroides* and *Peptostreptococcus productus.* Across all Wagner Ulcer Classification grades, *Clostridium* was the most prevalent anaerobe and was significantly associated with wound age and total leukocyte count of the patient with no difference in wound healing present between both groups at the end of 16 weeks. The authors concluded that it is should not be mandatory to supplement metronidazole in antibiotic regime for the treatments of DFUs.

Despite an array of studies on DFUs highlighted above the role microorganisms play in affecting the healing of a DFU lacks clarity and still represents an area in need of further investigation. In particular, more research is required on microbial interaction and virulence anaerobes and the significance of both planktonic and sessile microbes.

2 | CONCLUSION

Whilst the presence of microorganisms of a polymicrobial nature in DFUs is clearly evident there is still a large gap in knowledge over the distinct effect of anaerobic bacteria in the chronicity of DFUs and chronic wounds in general. This in part may be due to the varying methods of anaerobic microbiological isolation and evaluation and the models which are employed. What is intriguing is the evidence that supports the synergistic interactions between anaerobes and aerobes within *in vitro* biofilms. However, despite there being a small number of papers that identify the presence of biofilms within chronic wounds basic microbiological methods and even some molecular methods do not give us insight into the structure of a polymicrobial biofilm within these DFUs. The traditional physical architecture of a biofilm observed within *in vitro* biofilms, that is, mushroom structures are not considered to be found within *in vivo* biofilms. Consequently, studying and investigating the physical and chemical characteristics of “true to life” biofilms still remains a significant challenge. Despite this, our interpretations and extrapolations from *in vitro* biofilms has helped significantly to begin to develop information that is assisting with the management and study of biofilms of public health significance.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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