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Investigating the clinical importance of fungi in diabetic wounds

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Abstract

DFU is often manifested as a chronic open wound predisposed to invasion by many pathogenic and opportunistic microorganisms including bacteria and fungi. Impaired wound healing characterised by clinically infected ulcers can be a consequence of ineffective eradication of polymicrobial biofilms by antibiotic treatments, leading to limb amputation. A universal microbiome picture is necessary, especially for empirical therapy. Still, the dynamic of individual microbiomes can only be precisely depicted by periodic sampling of the wounds revealing the ongoing response to the antibiotic treatments.

Initially, standard microbiology culture data was analysed as part of the standard wound care for the patients. Of 306 samples with growth, 85.6% of the wounds were polymicrobial, and *Candida* was always found in mixed growth. Mixed skin, enterics and *S. aureus* were the topmost isolated organisms in descending order. Despite stratification of the wounds into different grades and stages according to the University of Texas wound classification systems, wounds were not found to be different. The data obtained from the standard culture showed that DFUs are predominated by bacteria from the skin and gut.

Secondly, Oxford Nanopore sequencing technology was utilised to scrutinize the 16S wound microbiomes of 349 extracted wound DNA. *Corynebacterium*, *Staphylococcus* and *Streptococcus* were the major taxa identified from the wounds. Moreover, *Finegoldia magna* was the most predominant obligate anaerobes identified, and commonly found with all *Candida* species. The wound diversity stratified according to the grades and stages showed no clustering. A subset of patients with multiple visits was then analysed for changes in microbiome as the wound progressed. Individual patient microbiomes were indeed unique.

An enhanced mycology culture was performed using Sabouraud dextrose agar (SAB) with chloramphenicol and CHROMagar™ for Malassezia to confirm the presence of fungi in the wounds. Thirty fungi isolated were *Candida* species mainly *C. parapsilosis*, *C. albicans* and *C. glabrata*. All fungi could form biofilms with *C. albicans* forming the most robust biofilms.

Lastly, real-world biofilm models grown from individual wounds were assessed for biofilm inhibition using Flucloxacillin, Fluconazole and a combination of both using concentrations typically found in the tissue. This model is based on an undefined microbial species found in the wounds.The bacteria and fungal load were quantified using 16S and ITS, respectively and changes in the 16S microbiome were assessed by nanopore sequencing. Antimicrobial treatments have minimal impact on the interkingdom biofilms that were formed consisting of bacteria and fungi.

In conclusion, the dynamics of diabetic wound microbiomes may represent interkingdom interactions between bacteria and fungi that exist as biofilms. This implicates the potential treatment of DFU, as systemic antibiotics alone may not be adequate to eliminate infection when fungi are present. Therefore, treatment targeting both infective aetiologies should be pursued to enhance the elimination of polymicrobial biofilms in chronic diabetic wounds.

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Author's Declaration

I declare that I have carried out the work described in this thesis unless otherwise acknowledged or cited, under the supervision of Professor Gordon Ramage and Dr. Jason Brown. I further declare that this thesis has not been submitted for any other degree at the University of Glasgow, or any other institution.

Ahmed Rafezzan Bin Ahmed Bakri

Abbreviations

AST	Antimicrobial susceptibility testing
BBWC	Biofilm-based wound care
CAP	Cold atmospheric plasma
CFE	Colony forming equivalence
CFU	Colony forming unit
CoNS	Coagulase negative *Staphylococcus*
CT	Community type
CV	Crystal violet
DFO	Diabetic foot osteomyelitis
DFU	Diabetic foot ulcer
ECM	Extracellular matrix
ESBL	Extended-spectrum beta lactamase
FEP	Functional Equivalent Pathogroup
FPPL	Fungal Priority Pathogen List
GI	Gastrointestinal
GPAC	Gram positive anaerobic cocci
ICU	Intensive care unit
IDSA	Infectious diseases society of America
ITS	Internal transcribed spacer
IWGDF	International Working Group on the Diabetic Foot
LSU	Large subunit
MALDI-ToF	Matrix Assisted Laser Desorption/Ionization - Time of flight
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MTPJ	Metatarsophalangeal joints
NGS	Next generation sequencing
NHS	National Health Service
OD	Optical density
OTU	Operational taxonomic unit
PAD	Peripheral arterial disease
PCoA	Principal Coordinate analysis
PCR	Polymerase chain reaction
PMA	Propidium monoazide
qPCR	Quantitative polymerase chain reaction
RA	Relative abundance
RLI	Royal Lancaster Infirmary
rpm	Revolutions per minute
RPMI	Rosewell Park Memorial Institute
SAB	Sabouraud dextrose agar
THB	Todd-Hewitt broth
UT	University of Texas wound classification system
UTI	Urinary tract infection
WHO	World Health Organization

YPD	Yeast peptone dextrose

1 General introduction

1.1 Introduction

The World Health Organization (WHO) has made a remarkable effort to address important fungal pathogens by introducing a fungal priority pathogen list (FPPL) in 2022 with clear aims to improve fungal diagnostic and public health involvement in research in response to the threat of fungal diseases (World Health Organization, 2022). Fungi can cause a broad spectrum of diseases from superficial to more invasive infections in susceptible individuals with risk factors such as diabetes, haematological malignancies, and intensive care unit (ICU) patients (Beed et al., 2014; G. D. Brown et al., 2024; Reddy et al., 2022). Diabetic foot ulcer (DFU) is one of the major complications from diabetes that can be difficult to heal and continuously exposed to a plethora of microorganisms including fungi. Yeast-like organisms such as *Candida* and filamentous fungi of hyaline and dematiaceous mould have been reported from DFU (Table 1.1). Moreover, colonisation by fungi is eight times higher in diabetic compared to non-diabetic individuals, putting them at risk of fungal infection (Saud et al., 2020). None of the wound classification systems has explicitly differentiated the clinical signs between bacterial and fungal infection indicating that diagnosis of fungal infection is often difficult due to the non-specificity of the signs of infection that can be observed clinically. Furthermore, the coexistence between fungi and bacteria in biofilms can potentiate further challenges in managing the infection as biofilms proved to be more resilient to drug treatment (E. Townsend et al., 2017). Despite the description of various multi-species biofilm models that highlight the importance of interspecies interaction, limited studies have included fungi to elucidate the real impact of interkingdom interaction in wound biofilms, particularly towards antimicrobial therapies. Besides that, the majority of the model only integrates well-established pathogenic microbes with high capacity to form robust biofilms while dismissing the potential role of normal microbiota.

This chapter set out to review the literature on the role of fungi in interkingdom wound biofilms and the implications of such interactions to the clinical interventions. Some contents of this chapter have been published in:

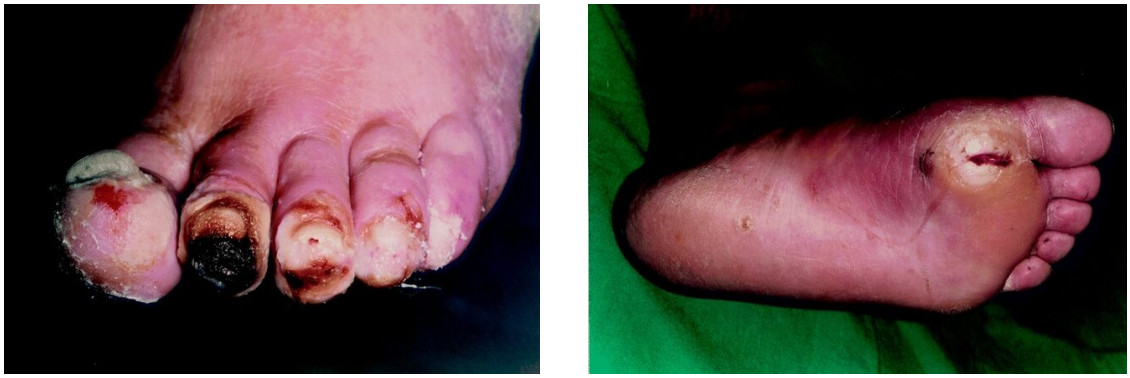
Short B, **Ahmed Bakri**, Abdullah Baz, Williams C, Brown J and Ramage G. 'There is more to wounds than bacteria: Fungal biofilms in chronic wounds'. Curr Clin Micro Rpt 10, 9 – 16 (2023).

1.2 Fungal disease in diabetics

1.2.1 Diabetic wound

Diabetic foot ulcer (DFU) is one example of a chronic wound that typically requires more than several weeks to several months to heal (Robertshaw et al., 2001; Sørensen et al., 2019). Normally, wound healing would progress in the order of haemostasis, inflammation, proliferation and remodelling but can be impeded at different stages including chronic inflammatory response leading to non-healing wound (Falanga, 2005; Q. Li et al., 2022). Formations of ulcers from the trauma could occur on any part of the foot. The most common area that is repeatedly exposed to pressure and commonly at risk of skin breakdown is mostly seen on plantar surface of the foot (Reiber et al., 1999).

About 20% of diabetic patients were estimated to undergo lower limb amputation due to infected DFU (Edmonds et al., 2021; McDermott et al., 2023). Table 1.1 summarizes several studies that indicate fungi in chronic wounds. Interestingly, fungal foot ulcer was also observed in two distinct syndromes. The fungi were observed in multiple ulcers that were located at the distal and subungual of the toes and the other form indicate an apparent macerated margin around the ulcer that was also not responding to the standard treatment (Figure 1.1) (Heald et al., 2001). Contradicting observations were reported between the association of fungal infection and length of diabetes. Diabetes duration of 12 years was found to have been associated with increased fungal infection (Sanniyasi et al., 2015). Similarly, the duration of ulcers could or could not be associated with fungal infection. Chelan *et al*. found no association with the mean duration of ulcers of ~7 weeks (Chellan et al., 2010) . In contrast, an ulcer duration of 7 – 14 days was observed to be correlated with higher fungal positivity (Sk et al., 2023). While DFU can remain superficial, it may also progress to diabetic foot osteomyelitis (DFO). The duration of the ulcer was not associated with the progress of DFU to DFO (Jaroenarpornwatana et al., 2023). Despite after healing, DFU has a tendency for recurrence. It was estimated that the recurrence could occur at 40%, 60% and 65% within 1,3 and 5 years respectively (Armstrong et al., 2017).



(a) (b)

Figure 1.1 Two distinct syndromes of ulcer caused by fungi. (a) Distal and subungual (b) Macerated ulcer (Heald et al, 2001).

1.2.1.1 Clinical significance

Patients who were diagnosed with diabetes in the UK have shown an increased trend with the majority of them were type-2 diabetes. Over 5 million people were estimated to have diabetes including the ones that have not been diagnosed yet. This high incidence also has incurred a minimum of £10 billion annually for the NHS to manage diabetes alone with most spending allocated for treating complications due to diabetes (https://www.diabetes.org.uk). Indeed, complications from diabetes can involve multiple organ-related diseases (Figure 1.2) (H.-Y. Jeon et al., 2022). Furthermore, predisposing factors such as peripheral arterial disease (PAD) can increase the chances of developing DFU and PAD was found in 50 % of DFU (Fitridge et al., 2024).

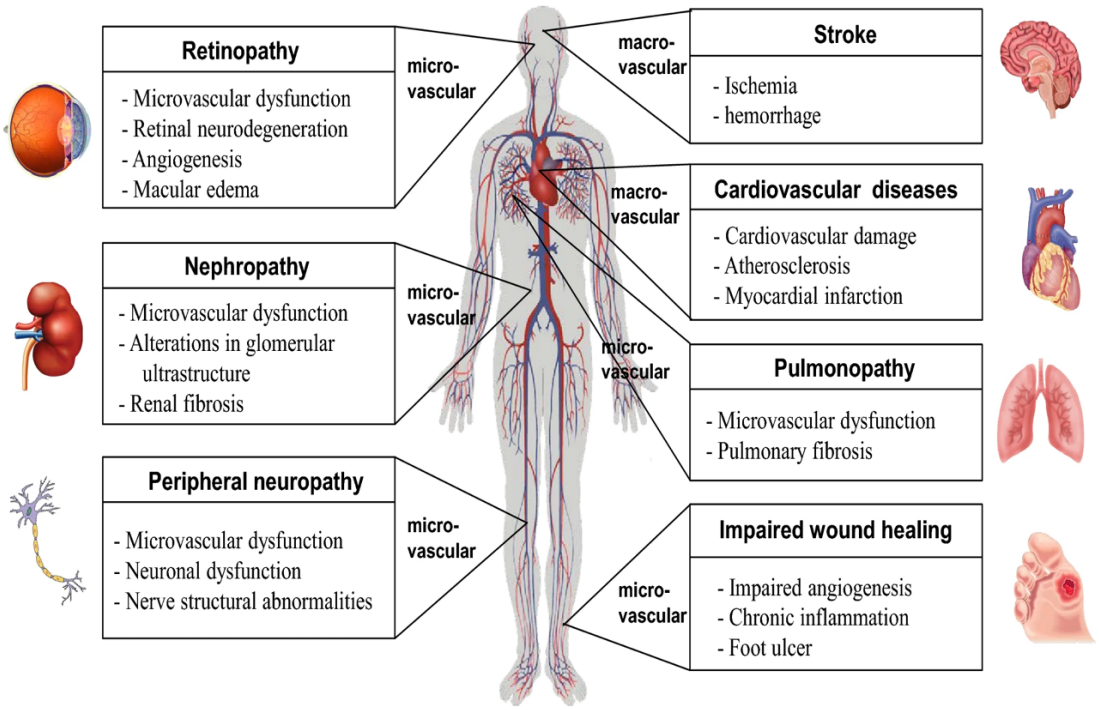


Figure 1.2 Complications from diabetes. (H. -Y. Jeon et al, 2022).

The global prevalence of DFU has been reported at 6.3% from a combined analysis of various reported studies from different regions including North America, Asia, Europe, Africa and Oceania (P. Zhang et al., 2017). It has also been estimated that diabetic patients will likely develop foot ulcers in their lifetime about 19 – 34% (Armstrong et al., 2017), an increased estimation from 15 – 25% of previous study (N. Singh et al., 2005). DFU-related complications predominantly infection occur at 50 – 60% (Edmonds et al., 2021; McDermott et al., 2023). A level of HbA1C between 7 -8% during treatment has been associated with increased wound healing (Xiang et al., 2019) while a higher level of > 8% is associated with amputation (Lane et al., 2020).

The amputation site may involve any part of the feet depending on the extent of tissue damage. However, the purpose of minor and major amputation is not the same. While minor amputation is aiming to restrict the spread of the infection while preserving the function of the limb, major amputation on the other hand is the last resort to be performed when all other treatment options have failed (Jeffcoate & van Houtum, 2004). Generally, minor amputation may involve removal of the subsection of the foot such as part of the toes while major amputation is a complete removal of the foot from either below or above the knee (Jeffcoate & van Houtum, 2004).

1.2.1.2 Wound characteristics

There are several classifications system that provides important information on the condition of the ulcer that will guide the clinicians in diagnosing the severity and the healing progress of the wound. The various ways of classifying the wounds include a combination of different aspects or clinical presentation of the ulcers that can be observed or measured, which are eventually presented as either scores or alphanumerical indexes corresponding to wound conditions (Monteiro-Soares et al., 2020). The most commonly observed aspect of the wound that also forms the fundamental part of the classification system is the depth of the wound. This has been represented as the extension or the extent of wound penetration into the tissue. Meggitt-Wagner wound classification is the earliest system that has included the wound depth to describe the extension of the ulcer, and the presence of gangrene or osteomyelitis based on grade 0 – 5 (Wagner, 1981). However, the system lacks identification of the presence of infection or ischemia.

On the other hand, the University of Texas wound classification system (UT) takes into account the depth of the wound and also the presence of infection and ischemia (Lavery et al., 1996). This system is based on a four-by-four table that indicates the depth and the severity of the wound represented by grade and stage, respectively. The system has also been validated by the same authors and wound was found to have poorer outcome the greater the grade and stages were (Armstrong et al., 1998). Other studies have found both Wagner and UT to be helpful at predicting the amputation outcome of the lower limb (B.-J. Jeon et al., 2017). In Scotland, UT was found to be more practical owing to its feasibility hence was selected and used as part of the standard care for classifying DFU (Stang & Young, 2018). The UT table is presented in Figure 1.3.

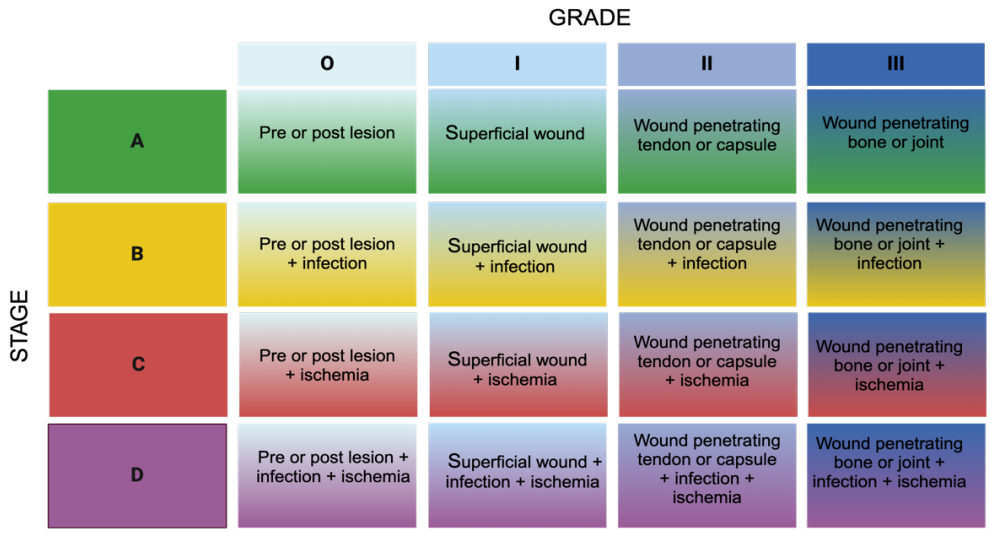


Figure 1.3 University of Texas wound classification system adapted from Lavery et. al, 1996. Created with Biorender.com

Another scoring system was developed that was based on six characteristics of the ulcers including site, ischemia, neuropathy, bacterial infection, area and depth (SINBAD) (Ince et al., 2008). This system is the modification to S(AD)SAD classification restricting to only two observations for each wound characteristic resulting in a maximum score of six. This system allows for assessment of the wound outcomes from different regions that can be influenced by different factors. Furthermore, a more comprehensive system that described the wound based on PEDIS (perfusion, extent, depth, infection and sensation) was developed by IWGDF (Schaper, 2004). This system's primary aim is to be applied for research purposes rather than assessing patient outcomes. Collectively, each system has its own criteria but also with limitations that may be applicable depending on the setting and purpose of use.

1.2.2 Other superficial infections

1.2.2.1 Dermatophytosis

Cutaneous fungal infections may involve different anatomical sites of the body such as skin, hair and nail (Chanyachailert et al., 2023; Howell, 2023). Fungal infection was found to be the most common cutaneous infection in patients with

diabetes compared to bacterial and viral infections (Demirseren et al., 2014; G. Romano et al., 1998; Shahzad et al., 2011). Of these, dermatophytes and *Candida* have often been reported. In a few comparative studies between healthy and diabetic subjects authors have found the prevalence of dermatophytes infection to be similar in both groups (BUXTON et al., 1996; Lugo-Somolinos & Sánchez, 1992; C. Romano et al., 2001). Furthermore, dermatophytosis was commonly seen as tinea pedis and onychomycosis in diabetic patients although there was no association with the glucose level (Oz et al., 2017). However, patient with type 2 diabetes with a higher HbA1c was found to be more prone to fungal infection (Eckhard et al., 2007). Many studies reported the presence of dermatophytes in diabetic individuals based on the isolation of the fungi from the feet, either associated with tinea pedis or onychomycosis, as these are common in diabetic patients (Lugo-Somolinos & Sánchez, 1992; Parada et al., 2013; Trovato et al., 2022). However, dermatophytes are a group of fungi that infect the keratinous layer of the skin not limited to feet but also other parts of the body, hair and nail although they may vary in prevalence to certain sites of the body (Moskaluk & VandeWoude, 2022; Tan & Joseph, 2004). Rarely, penetration of dermatophyte may surpass the dermis layer resulting in invasive infection that may be classified as Majocchi's granuloma, deeper dermal dermatophytosis, pseudomycetoma and disseminated dermatophytosis (Boral et al., 2018). Diabetes has been reported to be among the common predisposing factor for invasive dermatophytosis (Wang et al., 2021).

1.2.2.2 Superficial candidiasis

High glucose content in saliva has been shown to reflect high glucose content in the serum of diabetic patients (S. Kumar et al., 2014). The prevalence of *Candida* colonisation also varies between studies of diabetic and control groups that show up to 80% of diabetic patients were colonised by *Candida* (Soysa et al., 2006). While fungal colonisation in the gut of diabetic patients normally shows less than 10 CFU/g of faeces (Rodrigues et al., 2019), a high fungal load in the oral cavity (>50 colonies) was seen in diabetic patients with high glucose levels from mouth swabs (Mohammadi et al., 2016). In contrast, *Candida* CFU ranges from 0-5 CFU/ swab and 0 – 65 CFU/100 µL rinsing fluid both indicative of colonisation in non-diabetic individuals (Tooyama et al., 2015). As seen in colonised individuals, varying in *Candida* load was also similarly observed in people with oral *Candida*

infection (Patel, 2022). Despite the high fungal burden presented from previous studies, a meta-analysis does not show a difference in the tendency for diabetic patients to get oral candidiasis than the control (Martorano-Fernandes et al., 2020). While *C. albicans* was commonly isolated, mixed *Candida* infection forewarning for potential treatment inefficacy (Araiza et al., 2023).

Various fungal species including filamentous mould were found to be part of the vaginal mycobiome (Lehtoranta et al., 2021). *C. albicans* remains the most common species to be found in healthy women and its abundance reduces the diversity of other fungal species identified from the vagina (Lehtoranta et al., 2021). On the other hand, *C. glabrata* was found to be more predominant in patients with diabetes (D. Goswami et al., 2006; R. Goswami et al., 2000; Nyirjesy & Sobel, 2013; Sobel, 2007). About 70 – 75% of women in childbearing age were reported to have at least one VVC in their lifetime (Sobel, 2007). Moreover, recurrent VVC was also estimated to increase in diabetes patients globally (Denning et al., 2018). Symptoms including pruritus, dyspareunia and having discharge were similarly observed between diabetic and non-diabetics (D. Goswami et al., 2006).

1.3 Microbiome of DFU

1.3.1 Bacteriome

Identification of pathogenic organisms from infected DFU leads to the commencement of targeted treatment. Only culture-based identification is currently recommended to guide antimicrobial therapy according to the International Working Group on Diabetic Foot guideline (Senneville et al., 2024). Based on the same guideline, anticipation of the mixed organisms classified as gram-positive, gram-negative and anaerobes, together with certain clinical conditions, was proposed with beta-lactam or beta-lactamase inhibitor antibiotics for empirical treatment. Past studies on standard microbiology culture have identified common organisms to be associated with infected wounds. For example, a previous meta-analysis on the studies of DFU carried out in multiple countries has shown that *S. aureus* is the most common species isolated (Macdonald et al., 2021). This, however, assumes that all studies had the same chance of isolating the same organisms because of the different techniques used by the laboratories.

In a study of mild to severe DFU collected as part of the clinical trial, *Staphylococcus* is the most common genus identified, with *S. aureus* as the predominant species isolated (Citron et al., 2007). For gram-negative bacteria, *P. aeruginosa* is the most commonly identified among the other gram-negative aerobic bacteria. This study has also reported a higher identification of total anaerobes isolated compared to gram-negative aerobic bacteria, with gram-positive anaerobic cocci including *F. magna* being the most common. A few new species that have never been reported from DFU using any methods either culture or molecular have been isolated from culturomics (Jneid et al., 2018). These new species include *Raoultella ornithinolytica*, *Eubacterium massiliense*, *Eggerthella timonensis*, *Lachnoclostridium timonense*, and *Vaginiphocea massiliensis,* that were previously identified from gut microbiome study by their group (Lagier et al., 2016).

Due to the limitation of culture in elucidating a comprehensive picture of wound microbiome, a pioneer study by Dowd et al. has found that *Staphylococcus* is the most common genus found in DFU using partial ribosomal amplification pyrosequencing (PRAPS). In the same study, by using full ribosomal amplification, cloning and shotgun Sanger sequencing (FRACS), *S. aureus* was found to be the most common. PRAPS and FRACS were used to amplify 600 bp and 1500 bp or rRNA gene, respectively. There was variation in species reported from the two methods in terms of taxa resolution and the prevalence of certain taxa (Dowd, Sun, et al., 2008). Following this study, Dowd and colleagues carried out a similar survey of bacteria in DFU by using bacterial encoded FLX amplicon pyrosequencing (bTEFAP) and reported that *Corynebacterium* was more prevalent than *Staphylococcus* (Dowd, Wolcott, et al., 2008). They have also suggested that wound infection is caused by multiple organisms rather than by a single organism by introducing a concept of Functional Equivalent Pathogroup (FEP). In all of these FEPs, anaerobes are present in all clusters (Dowd, Wolcott, et al., 2008).

While infected DFUs have been a focus of many studies, DFUs with no sign of infection have been characterised from neuropathic DFUs. It was reported that these wounds were not predominated by either *Staphylococcus* or *Streptococcus* , and were instead colonised by anaerobes or Proteobacteria (S. Gardner et al., 2013). *S. aureus* was found to be the most predominant in the study, although the wounds were uninfected. Therefore, understanding of wounds with various

conditions could potentially offer more insight into the diversity of every grade or stage.

Apart from Dowd's group, which has clustered organisms according to the concept of co-existence, other studies have also attempted a similar approach. In a work by Loesche and colleagues, organisms were clustered based on the Community Type (CT), with CT3 and CT4 distinguished by the predominance of *Streptococcus* and *Staphylococcus aureus*, respectively (Loesche et al., 2017). On the other hand, CT1 and CT2 are not particularly different in the relative abundance of certain organisms and are more heterogenous. A frequent change in CTs was observed in wounds that heal faster. The taxa described in every CT were based on the sequencing of V1 to V3 of the 16S rRNA gene.

Non-random transition between community types can occur at 3.52 weeks on average based on the DFU samples collected fortnightly (Loesche et al., 2017). This important study shows that the shift in the DFU microbiome can occur over time. Moreover, it has been shown that less than 50% similarity of the species was observed between the two sampling times indicating variability of the microbiome (Tipton et al., 2017). Furthermore, by considering the relative abundance (RA) of the organism, low-abundance organisms of less than 1% RA can become dominant (>10% RA) in 16 -20% of the wounds. It is important to note that this study is not limited to only DFU, but also other types of chronic wounds (Tipton et al., 2017). Apart from changes in bacterial composition over time, fungal dynamic has also been studied, however, separately from the bacteria composition (L. Kalan et al., 2016).

1.3.2 Mycobiome

Despite having a general definition that covers all microorganisms within a biological niche, the word 'microbiome' is typically used to specifically reference bacteria, and separate terms such as virome and archaeome are now employed to specify between viruses and archaea, respectively. The mycobiome, which is specific to fungi, is an under-represented and under-appreciated area of microbiome research. For example, in the gut, fungi comprise less than 1% of all microorganisms (Qin et al., 2010). However, there are several arguments to suggest that fungi are more important than previously thought. Being more than

100 times larger than bacteria, fungi make up a considerable part of the collective microbiota biomass in addition to causing infections with high levels or morbidity and mortality.

As the first line of defence against foreign microorganisms, the skin is home to a myriad of bacteria, fungi and viruses (Findley et al., 2013). Using culture-dependant techniques, *Malassezia, Aspergillus* and *Candida* species are recognised as some of the most cultured fungi from the skin. This has then been confirmed using NGS (L. Kalan et al., 2016; L. Kalan & Grice, 2017). Despite being readily identified and isolated from healthy skin, the role that fungi play in chronic wounds and how they alter regular wound healing mechanisms is still debated within the literature. The role of fungi in health and disease is discussed, not just in chronic wounds but also in respiratory and oral infections (Pendleton et al., 2017). With this being said, Chellan and colleagues identified fungi infecting diabetic foot ulcers (DFUs) in 22% of patients, with *Candida* spp. being the most abundant (Chellan et al., 2010). More recently, culture-independent studies have identified fungi in up to 80% of samples (L. Kalan et al., 2016). Not only does this reinforce previous reports that culture-dependant techniques underestimate microbial colonisation and infection rates, but also indicates that previous predictions stating fungi are merely 'bystanders' to chronic wound infections is worth rethinking, as they likely play a more active role in infection.

The mycobiome composition is often determined by the body site much like its bacterial counterpart with, *Malassezia* spp. dominating most sites. However, the mycobiome of the foot and more moist areas is far more diverse which is comprised of genera such as *Candida, Aspergillus* and *Penicillium* (Findley et al., 2013). Findings by Kalan and co-workers (2016) showed that increased abundances of *Ascomycota* are significantly associated with longer healing times (L. Kalan et al., 2016). These reports show that the mycobiome may influence wound healing in a similar way to that of the bacterial microbiota, where increased bacterial diversity is associated with longer healing times (Loesche et al., 2017). A range of fungal reported from previous studies were summarised in Table 1.1.

Fungi have a reputation for being opportunistic pathogens, so combining an open wound with antibiotics (given as a first-line treatment option) and fungi colonising

the surrounding skin creates an ideal environment for fungal infection. Despite this obvious logic, fungi are often the subject of debate in disease biology as they are often thought to not play any active role in infection, though there is substantial evidence from the oral cavity that this is not the case (Delaney et al., 2018). An initial study in 2010 with the intentions of identifying fungal infection in wounds in diabetes patients found fungal infections in nearly 30% of cases, with *Candida* spp. being the most prevalent followed by members of the *Aspergillus* and *Trichosporon* genera (Gopi et al., 2010). For *Candida* spp., in particular, it has been shown that conditions in diabetic wounds and ulcers are optimal for inducing a shift from commensal to pathogen. Higher blood glucose levels result in *Candida* isolates displaying a higher degree of enzyme activity, which is hypothesised to make these organisms more virulent (Fatahinia et al., 2015). These clinical studies highlight the importance of considering fungi in chronic wounds and should also drive consideration for antifungal therapy.

Table 1.1 Fungal species detected from various studies.

Study	Fungal species from DFU
(L. Kalan et al., 2016)	*Cladosporium herbarum, C. albicans, Family Nectriaceae ,C. parapsilosis, A. cibarius, Epicoccum nigrum, Penicillium species, Leptosphaerulina chartarum, Penicillium bialowiezense, Gibberella zeae, Hypocreales species, Order Capnodiales, Trichosporon asahii, Trichosporon species, Rhodosporidium diobovatum*
(M. Wu et al., 2018)	Fungi
(João et al., 2021)	*Fusarium oxysporum*
(Öztürk et al., 2019)	*Fusarium species, Fusarium solanii, Trichosporon asahii, C. albicans, C. glabrata, C. parapsilosis, C. krusei, C. lipolytica*
(D et al., 2013)	*Scedosporium apiospermum*
(Musyoki et al., 2022)	*C. albicans, C. lusitaniae, C. dubluniensis, C. parapsilosis, C. glabrata, C. tropicalis, C. famata, Penicillium species, Aspergillus species, Microsporum species, Trichophyton species*
(D. Kumar et al., 2016)	*C. tropicalis, C. albicans, C. krusei, C. parapsilosis, C. glabrata*
(Arun et al., 2019)	*C. parapsilosis, C. albicans, C. auris, Trichosporon species*
(Gitau et al., 2011)	*C. boidinii, C. famata, C. guilliermondii, C. parapsilosis, C. tropicalis, C. albicans, Pichia ohmeri, Rhodotorula mucilaginosa*
(Hassan et al., 2019)	*C. albicans*
(Kandregula et al., 2022)	*C. albicans, C. tropicalis, C. parapsilosis, C. lusitaniae, C. glabrata, Altenaria species, Penicillium species, Trichophyton species*

(Dowd et al., 2011)	*C. parapsilosis, C. albicans, C. orthopsilosis, C. dubliniensis, C. tropicalis, T. mentagrophytes*
(Bansal et al., 2008)	*C. tropicalis, C. albicans, C. guillermondi, A. flavus, A. niger, Fusarium species*
(Heald et al., 2001)	*C. parapsilosis, C. humicola, C. albicans, C. tropicalis, C. glabrata*
(Mehra et al., 2017)	*Aspergillus species, Candida species, Fusarium species, Trichophyton species*
(Raza & Anurshetru, 2017)	*Candida species, Trichosporon species, A. niger, A. flavus, A. fumigatus, Fusarium, Trichophyton rubrum, Trichophyton species, Penicillium species, Acremonium*
(Chai et al., 2021)	*C. albicans, C. tropicalis*
(Kalshetti et al., 2017)	*C. albicans, C. tropicalis, C. glabrata, T. mentagrophytes, T. rubrum, A. fumigatus*
(Abilash et al., 2015)	*C. albicans, C. tropicalis*
(Chellan et al., 2010)	*C. parapsilosis, C. tropicalis, T. asahii, C. albicans, Aspergillus species, C. guilliermondii, C. glabrata, Fusarium species, Candida sake, Zygosaccharomyces species, Kodamaea ohmeri, C. globosa, C. krusei, Penicillium species, C. lusitaniae, C. famata, C. melibiosica*
(Nair et al., 2006)	*C. albicans, C. tropicalis, C. parapsilosis, C. guillermondii, C. krusei, Trichosporon cutaneum, T. capitatum ,Aspergillus flavus,A. fumigatus, Fusarium solani, P. marneffei, Basidiobolus ranarum*
(Fata et al., 2011)	*C. albicans, C. tropicalis, C. parapsilosis, C. glabrata, C. krusei, Candida species, T. mentagrophytes, Rhodotorula species, Acremonium species, Scopulariopsis species, A. fumigatus*
(W. J. Lee et al., 2014)	*Trichophyton rubrum*

(Sujatha et al., n.d.)	*C. albicans, C. tropicalis, C. glabrata, Trichophyton mentagrophytes, Trichophyton rubrum, Aspergillus fumigatus*

1.3.3 Challenges in studying mycobiome

As previously stated, there are significant discrepancies between culture-dependant and independent methodologies in microorganism identification (Dickson et al., 2014). Despite the increased sensitivity that comes with molecular diagnostics such as NGS, the application of this to the mycobiome as a diagnostic method does not come without its downfalls. Challenges in mycobiome research come at many stages from sample processing to the final data analysis stages. For example, some challenges are common across micro- and mycobiome studies such as untimely processing or freezing of samples and repeated freeze-thawing of samples can influence microbiota diversity (Cuthbertson et al., 2015; Nilsson et al., 2019). Additionally, harsher methods of cell lysis are required due to the robust nature of the fungal cell wall, therefore, choice of DNA extraction method is important. For instance, chemical/enzymatic lysis can increase DNA yields whilst favouring the lysis of yeasts (e.g. *C. albicans*) whereas physical lysis produces higher DNA yields in filamentous fungi such as *A. fumigatus* (Fredricks et al., 2005; Vesty et al., 2017). Issues can also arise in the data analysis and bioinformatic stages with incomplete fungal reference databases, leading to large numbers of unclassified operational taxonomic units (OTUs)(Mac Aogáin et al., 2019). Several other factors contribute towards the difficulties of mycobiome research. However, the minutia of details behind these which fall out with the scope of this review have been extensively reviewed by Tiew and colleagues (Tiew et al., 2020).

1.4 Interkingdom interaction in DFU biofilm

1.4.1 Bacterial biofilms

Biofilms have been classically defined as a community of cells adhered to a surface, encased in a self-produced extracellular matrix (ECM). Microorganisms that transition from free-floating to sessile, biofilm cells exhibit increased antimicrobial tolerance and virulence compared to their planktonic counterparts (Di Domenico et al., 2017; Høiby et al., 2010). This biofilm-associated phenotype can often complicate the management of DFUs and chronic wounds. However, it is worth noting that biofilms *in vivo*, particularly those in chronic wounds differ from the traditional 'mushroom-like' structure which was first described in *Pseudomonas* grown under continuous-flow conditions where-by bacterial cells adhere and multiply forming a 'stalk' which then blooms outwards, creating a shape reminiscent of a closed-cup mushroom. Despite this, they still possess the traits normally associated with biofilms such as increased virulence and antimicrobial tolerance, which comes as a result of ECM production (Bjarnsholt et al., 2008). Biofilms in chronic wounds are adhered to one another more so than they are bound to the host or one another and this creates a smaller, aggregation of cells between 5 and 200 μm in diameter (Bjarnsholt et al., 2013). These non-surface-attached aggregates are now well described and are part of a reconceptualised thinking of the biofilm lifecycle, though notably excluding the role of fungi (Sauer et al., 2022).

Early studies that focused on biofilm infections in chronic wounds gave particular attention to bacteria such as *Pseudomonas aeruginosa*, an opportunistic pathogen that is not often found as part of the healthy skin microbiome, but can be readily isolated from chronic wounds (Oh et al., 2016; K. Smith et al., 2016). These studies showed that *P. aeruginosa* also formed bacterial aggregates within the host and utilised an arsenal of virulence factors regulated by the LasR quorum sensing system (Bjarnsholt et al., 2008). While many studies have focused on single species biofilms it is important to note that the chronic wound microbiome is a complex entity, therefore studies must give attention to multi-species biofilms. To date, several multi-species biofilm models exist to study chronic wounds, all of which favour *S. aureus* and *P. aeruginosa* (Table 1.2). A number of these make use of the Lubbock chronic wound biofilm (LCWM) model, which utilises Bolton Broth,

plasma and lysed blood (Sun et al., 2008). The benefit to this growth media is that biofilms formed by coagulase positive organisms such as *S. aureus* result in the formation of aggregates which mimic the biofilm phenotype observed *in vivo* (Sun et al., 2008).

Previous studies have highlighted the increased recalcitrance to antimicrobials of multi-species biofilms compared to single-species biofilms. In a rat model, higher rates of infection were observed from a dual-species inoculum consisting of *S. aureus* and *P. aeruginosa* (Hendricks et al., 2001). Similarly, the anaerobic bacteria, *Prevotella bivia* increases *S. aureus* pathogenicity in a murine infection model (Mikamo et al., 1998). A study by Dalton and colleagues showed similar findings when using a multi-species bacterial biofilm model to interrogate inter-species interactions. These complex multi-species biofilms, containing *Enterococcus faecalis*, *Finegoldia magna*, *P. aeruginosa* and *S. aureus*, resulted in healing impairment whilst remaining viable over a period of 12 days. These authors reported a decrease in wound healing and increased antimicrobial tolerance to treatments compared to single species biofilm counterparts (Dalton et al., 2011).

Although the addition of multiple species to biofilm models increases their relevance, it is important to note the utilisation of appropriate growth media and substrates to effectively mimic *in vivo* conditions (Cornforth et al., 2018). A recent publication evaluated the role of dual-species biofilms formed by *P. aeruginosa* and *S. aureus* in chronic wounds using a novel, layered substrate (X. Chen et al., 2021). To create this model, firstly, a subcutaneous fat layer was created and this was followed by a surrogate dermis layer before the addition of bacterial inoculum. Following bacteria growth, this model more accurately represented the biofilm phenotype often seen *in vivo* and supported viable bacteria for up to 9 days, which could be used to test antimicrobial washes and dressings. Authors showed these dressings only exhibited a mild anti-biofilm effect, which agrees with clinical findings and highlights the importance of using appropriate substrates and conditions when studying disease biology *in vitro* (X. Chen et al., 2021; Schwarzer et al., 2020). This study makes a large step in the right direction concerning the development of accurate and reproducible chronic wound biofilms, though remarkably these models fail to take into account the role of fungi.

1.4.2 Fungal biofilms

Yeast-like organisms known to form biofilms encompassed medically important species such as *Candida*, *Cryptococcus*, *Trichosporon* and *Malassezia* species (Desai et al., 2014; Martinez & Fries, 2010). On the other hand, mould or filamentous fungi biofilms are a problem caused by but not limited to *Aspergillus fumigatus*, *Fusarium* and dimorphic moulds such as *Coccidioides immitis* and *S. schenckii* (Martinez & Fries, 2010; Sánchez-Herrera et al., 2021). For filamentous moulds such as *Aspergillus* and dermatohpytes, biofilm formation is implied by the term fungal mass when found in the lung and nail, respectively (Burkhart et al., 2002; Harding et al., 2009; Ramage et al., 2011). Another characteristic, such as the formation of sporodochia by *Fusarium* on necrotic tissue, indicates the colonisation of the fungi on superficial wounds (M. Smith & McGinnis, 2005).

Currently, there is no diagnostic method that can directly detect biofilms in clinical samples. Wet and dry surface biofilms have both been demonstrated in clinical settings (Maillard & Centeleghe, 2023; Vickery et al., 2012). Although most biofilms have been extensively studied in hydrated form, some fungi can form biofilms on dry surfaces. This includes the persistency of *C. auris* in healthcare settings following 14 days of drying showing a higher burden of biofilms compared to *C. albicans* (Horton et al., 2020).

Fungal biofilms are similar to bacterial biofilms in terms of developmental stages that begin with attachment to the surface, matrix production and finally maturation stage as simply described (Ramage et al., 2011). When compared to yeast-like such as *Candida albicans*, biofilms formed by filamentous mould like *A. fumigatus* follow the same stages (Jyotsna et al., 2001; Ramage et al., 2011). *A. fumigatus* biofilms can vary in thickness depending on the inoculum concentrations, with higher density conidia resulting in reduced depth of biofilm (Mowat et al., 2007). At 10^6 conidia/mL, biofilm was found to be at 117 µm. When compared to *C. albicans* at 10^7 cells/mL, biofilm depth was greater in the thickness at about 450 µm (Jyotsna et al., 2001). Moreover, *C. albicans* biofilm was observed with dense hyphae at the top layer compared to yeast cells on the basal layer (Jyotsna et al., 2001).

Biofilms formed by thermally dimorphic fungi have been studied in both yeast and filamentous forms (Brilhante et al., 2015; Sánchez-Herrera et al., 2021). The development of *S. schenckii* filamentous biofilm into mature form took about 120 hours and was relatively slow compared to biofilm growth in yeast (Brilhante et al., 2018, 2019). The structure of the mature biofilms was found to be primarily with hyphae and conidia when filamentous biofilm was grown (Brilhante et al., 2018). In contrast, yeast cells with blastoconidia were observed in yeast biofilm (Brilhante et al., 2019).

1.4.3 Polymicrobial biofilms

With a myriad of different organisms inhabiting chronic wounds, understanding the interactions between these organisms is crucial in understanding their roles in disease. There are numerous bacteria-bacteria interactions that take place within wound environments which have been well documented elsewhere (Durand et al., 2022). However, fungal-bacterial interactions are less well known. Many studies focusing on these interactions do so in the context of oral or respiratory disease, meaning not only should findings be translated to chronic wounds with caution, but also more studies must study interkingdom dynamics in a chronic wound model.

Candida – Staphylococcus interactions

Interactions between fungi and bacteria found in DFU infections may drive antimicrobial tolerance and virulence (J. L. Brown et al., 2022). For example, a well-studied interkingdom relationship between *C. albicans* and *S. aureus*, two organisms often found in DFUs and chronic wounds, is known to increase *S. aureus* tolerance to antibiotics by increasing extracellular DNA production and fungal ECM components as well as increasing virulence by upregulating the *agr* quorum sensing pathway, resulting in increased toxin production (A. et al., 2019; F. et al., 2016; Vila et al., 2021). This increase in tolerance and virulence is reciprocal which has been confirmed by *S. aureus* upregulating *C. albicans* biofilm and virulence genes (Figure 1.4). The presence of *C. albicans* within an interkingdom chronic wound biofilm was identified as a driving force behind antimicrobial tolerance, highlighting the importance of fungi in wound biofilms and why targeting the

fungal scaffold within these biofilms may yield more positive treatment outcomes (E. Townsend et al., 2017).

Candida – Streptococcus interactions

Another bacterial genus commonly found in the chronic wound microbiome is *Streptococcus* (K. Smith et al., 2016). *Streptococcus agalactiae* is the most abundant species of *Streptococcus* found in chronic wounds and unlike many other members of the *Streptococcus* genus, interactions between this bacterium and *C. albicans* are still subject to debate with some reports stating *S. agalactiae* inhibiting *C. albicans* hyphal formation by repressing expression of HWP and EFG (Yu et al., 2018). However, others report that *C. albicans* increases *S. agalactiae* colonisation in a murine infection model whilst also documenting the presence of hyphae in these infections (Shing et al., 2020). Additional studies are in agreement that interactions between *C. albicans* and group B *Streptococci*, such as *S. agalactiae*, are beneficial for the organisms with close binding occurring between fungus and bacterium that likely promotes bacterial colonisation and virulence (Figure 1.4) (R. et al., 2018). It is important to note that the absence of hyphae may not necessarily come as a detriment to *C. albicans*. For example, as hyphae are highly immunogenic, maintaining a budding yeast phenotype may help promote chronic colonisation in the wound bed. This is in line with a recent study whereby *P. aeruginosa* wound isolates were defective in virulence functions suggesting such factors are not required for microbial fitness in wounds (Morgan et al., 2019).

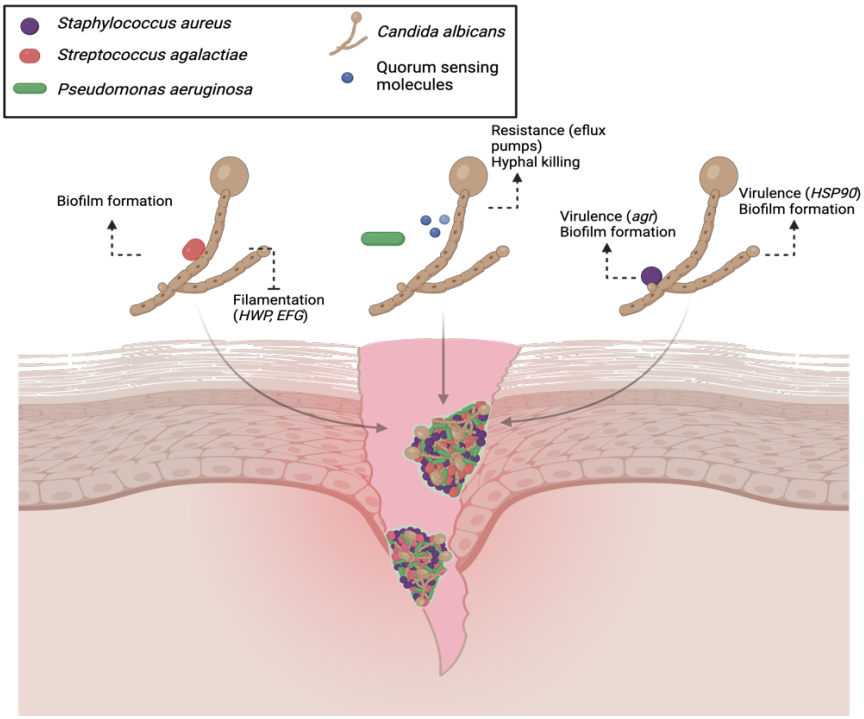


Figure 1.4 Interkingdom biofilm interactions. The interaction between *C. albicans* and bacteria of interest is summarised to highlight their implications. Created with Biorender.com

Candida - *Pseudomonas* interactions

An interkingdom consortium that is also commonplace within wound environments is that of *C. albicans* and *P. aeruginosa* (Dhamgaye et al., 2016). The interactions that occur between these two organisms are more complex than that of *Staphylococcus* as interactions primarily happen indirectly via quorum sensing molecules rather than direct binding and antagonistic and synergistic interactions can seemingly take place simultaneously. For example, *P. aeruginosa* induces an upregulation of *C. albicans* stress pathways, killing hyphal cells (Fourie et al., 2021; Hogan & Kolter, 2002). While on the other hand, using transcriptomic and proteomic approaches Bandara and colleagues showed that *P. aeruginosa* quorum sensing also promotes fluconazole resistance in *C. albicans* through upregulation of efflux pumps and ergosterol biosynthesis (Figure 1.4) (Bandara et al., 2020). Despite many antagonistic interactions taking place *in vitro*, their behaviour *in vivo* appears more synergistic with ventilator-associated pneumonia patients colonised by *Candida* are at a much greater risk of *P. aeruginosa* infection (Hamet et al., 2012).

Modelling interkingdom wound biofilms

There is growing evidence to support the notion that bacteria and fungi influence one another's behaviour, which in turn can have a clinical impact (Peleg et al., 2010). However, these interactions are best studied *in vitro* to gain a deeper understanding of the antagonistic and synergistic virulence potential of interkingdom interactions. Therefore, it is important to model these infections to accurately study the functionality of the chronic wound microbiome. Although a number of research groups have developed multi-species biofilm models to study microbial dynamics within chronic wounds (Table 1.2), these are largely devoid of fungi within their composition.

A multi-species biofilm model containing *C. albicans* and the prolific wound pathogens, *S. aureus* and *P. aeruginosa*, was initially described by our group and was one of the first to consider fungi in these models. The data showed that the presence of *C. albicans* was responsible for driving the recalcitrant nature of the biofilm, where antimicrobial treatments merely influenced biofilm composition rather than reducing overall biofilm biomass (E. Townsend et al., 2017). Although these data highlight the importance of fungi within wound infections, it is limited to a small number of species. We therefore enhanced the complexity of the model to a complex, 11-species interkingdom biofilm model adjacent to 3-dimensional tissue (J. L. Brown et al., 2022). This biofilm consortium more accurately models wound conditions by the inclusion of additional aerobic and anaerobic bacteria. Data from this study showed that although challenging wound biofilms with antiseptics can significantly reduce viable biofilm cells, a considerable portion of the biofilm remains. The residual biofilm cells that were able to persist following antiseptic exposure presented differential stimulatory effects within the epidermis model, with H_2O_2 and povidone iodine being perhaps the more appropriate antiseptics due to their more effective immune-modulatory effects (J. L. Brown et al., 2022). Additionally, this study highlighted how differing atmospheric O_2 concentrations can influence the overall composition of the biofilm with *C. albicans* dominating biofilms grown in O_2 and CO_2 conditions, whereas *Staphylococcus hominis* dominated biofilms growing in anaerobic environments. These data further stress the point made above, in that the conditions that biofilm models are constructed should be carefully considered to effectively replicate *in vivo* conditions.

Table 1.2 Multi-species wound models. (Short et al, 2023)

Authors	Bacterial/Fungal spp	Substratum	Media
(Ammons et al., 2011)	MRSA, *Pseudomonas aeruginosa*	Porous membrane	10% brain heart infusion broth
(J. L. Brown et al., 2022)	*Candida albicans, Staphylococcus hominis, Peptoniphilus gorbachii, Corynebacterium simulans, Streptococcus agalactiae, Anaerococcus vaginalis, Pseudomonas aeruginosa, Staphylococcus aureus, Prevotella buccalis, Finegoldia magna* and *Porphyromonas asaccharolytica*	Cellulose matrix	50% horse serum hydrogel
(X. Chen et al., 2021)	*Staphylococcus aureus* and *Pseudomonas aeruginosa*	Layered chronic wound biofilm model	25% Tryptic soy broth and 0.5% agar
(Dalton et al., 2011)	*Staphylococcus aureus, Pseudomonas aeruginosa, Finegoldia magna* and *Enterococcus faecalis*	Pipette tip	Bolton broth, 50% plasma and 5% lysed horse blood
(Di Giulio et al., 2020)	*Staphylococcus aureus* and *Pseudomonas aeruginosa*	Pipette tip	Brucella Broth, 0.1% agar, 50% plasma, 5% horse erythrocytes and 2% foetal bovine serum
(Gounani et al., 2020)	*Staphylococcus aureus* and *Pseudomonas aeruginosa*	Cell-derived matrix	Tryptic soy broth+ glucose+ NaCl+ foetal bovine serum
(He et al., 2021)	*Staphylococcus aureus* and *Pseudomonas aeruginosa*	Pipette tip	Tryptic soy broth, 50% plasma and 5% lysed horse blood
(Kim & Izadjoo, 2015)	*Staphylococcus aureus* and *Pseudomonas aeruginosa*	Glass cover slip	Poloxamer hydrogel
(Kucera et al., 2014)	*Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtillis* and *Enterococcus faecalis*	Pipette tip	Bolton broth, 1% gelatine, 50% plasma and 5% freeze-thawed porcine erythrocytes

(Sojka et al., 2016)	*Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus agalacticae* and *Enterococcus faecalis*	Pipette tip	Bolton broth, 1% gelatine, 50% plasma and 5% freeze-thawed porcine erythrocytes
(Su et al., 2020)	MRSA, *Pseudomonas aeruginosa*	Human skin	Tryptic soy broth
(Sun et al., 2008)	*Staphylococcus aureus, Pseudomonas aeruginosa* and *Enterococcus faecalis*	Pipette tip	Bolton broth, 50% plasma and 5% lysed horse blood
(Touzel et al., 2016)	*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsellia pneumoniae* and *Enterococcus faecalis*	CDC biofilm reactor (polypropylene coupons)	Bolton broth, 50% plasma and 5% lysed horse blood
(E. M. Townsend et al., 2016)	*Candida albicans, Pseudomonas aeruginosa* and *Staphylococcus aureus*	Cellulose matrix	50% horse serum hydrogel
(Woods et al., 2012)	*Staphylococcus aureus, Pseudomonas aeruginosa* and *Clostridium perfringens*	Glass	Brain heart infusion broth and 5% adult bovine serum

1.5 Laboratory diagnosis of DFU infection

1.5.1 Conventional culture

Reflecting on early studies of diabetic foot infection, microbiology culture has long been used to identify pathogenic bacteria from wound samples (Sapico et al., 1984; Sharp et al., 1979). One of the important roles of culture is to isolate viable organisms that potentially causing the infection. The capacity of culture to yield a diverse range of aerobic, anaerobic and fungi depends on factors such as the culture conditions used during the incubation and the various media types (Bonnet et al., 2020). In addition, the isolation of the viable organism will allow for antibiotic susceptibility testing to be performed leading to the detection of resistant organisms (Giuliano et al., 2019). Currently, microbiology culture has been included in the management guideline of DFU as a recommended method to diagnose the infection (Lipsky et al., 2020). With standard culture, the causative of the likely pathogenic organism can be determined resulting in targeted antibiotic treatment.

Isolation of multiple organisms from DFU culture is commonly reported with an average of 1.5 organisms can be isolated from a single culture (Macdonald et al., 2021). Organisms recovered can be broadly categorised into aerobe and obligate anaerobe. About 71% of aerobic bacteria are predominantly isolated compared to only ~29% of anaerobes (Citron et al., 2007). On the other hand, fungi are scarcely isolated from the infected wound with only about 3% of the total isolates (Ge et al., 2002). The diversity of organisms from culture also depends on the type of specimen collected, with anaerobes more often isolated from tissue samples (Citron et al., 2007). Although culture results from tissue samples are more influential and favourable by clinicians in deciding to change antibiotics, there are varying concordances in identifying organisms from swabs and tissue samples. In one study, an average of 3.7 organisms were isolated from the tissue samples compared to only 3 organisms by swab samples taken at the same time (Kelkar & Kagal, 2004). In another study, swab and tissue samples were comparable during the initial culture with averages of 2.34 and 2.07 isolates, respectively (Pellizzer et al., 2001).

While the identity of the causative pathogen from infected wounds is vital, the measurement of microbial load or quantitative culture remains debatable. A bacteria count of more than 10^6 CFU/mL has been associated with infection of shorter duration wounds (Soldevila-Boixader et al., 2022). In the same study, the fungal burden of *Candida albicans* and *Aspergillus* species was found at less than 10^6 CFU/mL in a total of three samples but has not been addressed for further analysis. In contrast, Gardner et. al have shown that a high microbial load of more than 10^6 CFU per gram of tissue is more associated with longer duration ulcers (S. E. Gardner et al., 2009). Additionally, signs of infection could not be associated with high or low microbial load. The more species of bacteria been isolated, the higher the CFU count been reported (Demetriou et al., 2013). However, generalisability from this study might not always be true as bacterial count may not always be evenly distributed among the species in the wound. An increase in bacterial load has been associated with delayed wound healing with a CFU of 10^4 was shown to have no substantial progress following four weeks of assessment in 13% of patients (n=32) (Xu et al., 2007). However, this study only included the aerobic culture from the wound fluid. Apart from that, a semi-quantitative count of the organisms from chronic wound swabs was shown to differentiate between colonisers and pathogens based on grading within the area of the streaking quadrant with 2+ and 3+/4+ indicating colonisers and pathogens, respectively (Banu & Vanaja, 2018). However, the count was only observed for aerobic bacteria. In addition, the heterogenous distribution of the organism in the wound is another concern when measuring the microbial load with only 63% concordance reported between peripheral and central lesions of the same ulcer of other wound aetiology than diabetic wound (Bowler et al., 2001; Sapico et al., 1986).

Unlike most bacteria, fungi often grow slower, especially for filamentous mould including dermatophytes (Zhu et al., 2021). According to EORTC/MSG consensus criteria, culture is strong evidence to prove fungal infection (Donnelly et al., 2020). Targeted isolation of fungi includes the use of medium supplemented with broad spectrum antibiotics such as SAB with chloramphenicol or gentamicin to inhibit or suppress the overgrowth of bacteria (Chincholikar & Pal, 2002; Kandregula et al., 2022; Musyoki et al., 2022). Additionally, medium added with cycloheximide has also been used to inhibit the growth of saprophytic fungi (Kandregula et al., 2022; Mehra et al., 2017). Normally, prolong incubation ranges

from 1 to 6 weeks are performed for fungal culture to cover for slow-growing mould (Chincholikar & Pal, 2002; Kandregula et al., 2022; Musyoki et al., 2022). Despite the use of enhanced methods to grow fungi, certain fungi including *Malassezia* species are hard to grow. *M. furfur* in particular is a lipophilic organism that is commonly found on the skin. Therefore, a culture medium to include this organism while studying DFU is essential to rule in possible causative agents contributing to non-healing wounds.

Laboratory capacity to grow and isolate broad range of bacteria and fungi species is critical as this will allow for antimicrobial susceptibility testing (AST) to be carried out. Furthermore, accurate identification of species is paramount to ensure appropriate selection of antibiotics or antifungals to be tested. In cases where polymicrobial infections are observed, careful selection of antibiotics to cover for broader range of bacteria can be done. Moreover, changes to an antibiotic treatment can be made based on the culture and sensitivity result (Senneville et al., 2024). Despite current improvements in identifying bacteria such as the use of MALDI-ToF , prior isolation of the organisms is still limited and reliant on laboratory conditions such as media selection (Złoch et al., 2021).

1.5.2 Next generation sequencing

To date, a few sequencing platforms have been utilised to study the microbiome of DFU. These include 454-pyroseqeuncing, Ion-torrent, Illumina and recently by using Oxford Nanopore technology (Dowd, Sun, et al., 2008; S. Gardner et al., 2013; Jnana et al., 2020; L. Kalan et al., 2016; Moon et al., 2021; K. Smith et al., 2016). All these studies are based on the detection of 16S rDNA for bacteria and ITS region for fungi in a study by Kalan et. al. The rapid detection of pathogens from Nanopore technology has been found valuable in the pathogen detection of other diseases (Q. Huang et al., 2021; Tanaka et al., 2019).

Prior findings from the studies that utilized sequencing technology have reported extensive detection of organisms that are not isolated by standard microbiology culture (Jneid et al., 2017). Of these, anaerobic bacteria are the most frequently missed (Moon et al., 2021). Despite a range of culture media combinations used that have yielded over 200 bacterial isolates, there were only 18 species identified following Maldi-ToF identification which include gram-positive and gram-negative

bacteria (Złoch et al., 2021). An enrichment step before culturing has increased the identification to 53 species of bacteria obtained from about 30000 isolates with an average of 698 isolates per sample (Jneid et al., 2018). Despite this increase, certain isolates without the identification from MALDI-ToF were still relying on the 16S identification. When sequencing was utilized, taxa detection and identification increased as expressed in Operational Taxonomic Unit (OTU). The number of OTUs detected varies among studies. For example, 276 and 1139 OTUs that are equivalent to various genera and species have been reported (Jnana et al., 2020; Malone, Johani, et al., 2017).

Furthermore, the sensitivity of the sequencing technology has shown higher detection of polymicrobial infection than the culture (Y. Huang et al., 2022; Lipof et al., 2021). Moreover, culture negative often results in positive detection by sequencing (Y. Huang et al., 2022). This usually involves anaerobic organisms that fail to grow. By utilising sequencing technology, relative abundances of bacteria can be measured together with taxonomic identification. It has been shown that 47% of the bacteria were cultured when the relative abundance was more than 10% and decreased to 15% for bacteria within 1-10% abundance(Rhoads et al., 2012).

Generally, Illumina is one of the most commonly used platforms in studying chronic wound microbiome with Nanopore reported in one study (Morsli et al., 2024). Comparison of microbiome studies between Illumina and Nanopore of different sample origins have shown comparable identification at the genus level (Heikema et al., 2020; Winand et al., 2020). Although relatively new, Nanopore is preferred compared to Illumina when taxonomic identification to species level is desired (Szoboszlay et al., 2023). A comparison between Nanopore and Illumina sequencing from a diabetic heel ulcer has shown higher resolution to species level that was limited with Illumina sequencing (Sloan et al., 2019). In contrast to Illumina, longer reads are one of the advantages of using Nanopore which allows for the whole 16S rDNA region to be sequenced leading to higher resolution of taxonomic identification (Benítez-Páez et al., 2016; Kai et al., 2019). Despite of higher error rate, Nanopore has continuously undergone improvement to increase the read accuracy.

While NGS has been widely used to sequence the bacterial genome, only Kalan et. al has identified fungi from DFU by targeting ITS1 region using Illumina (L. Kalan et al., 2016). However, targeting the ITS1 alone has been shown to result in less discriminatory power for correct taxonomic identification (Ohta et al., 2023). In the same study, a recent use of Nanopore sequencing with a longer target sequence covering both ITS regions and LSU has been shown to improve fungal species identification. Nevertheless, certain species were still unable to be identified despite a long target sequence of DNA (Ohta et al., 2023). Therefore, the detection of fungi requires further work to improve the coverage of fungal identification before can be applied in various mycobiome studies.

1.6 Treatment of DFU

1.6.1 Antimicrobials

As much as 45% of DFU were prescribed with antibiotics due to the presence of clinical signs of infection during the initial visit or the concern over the risk of infection (Guest et al., 2018). According to IWGDF/IDSA guidelines, antibiotics should not be used in uninfected DFU (Senneville et al., 2024). However, in the same guideline, suggested antibiotics treatment has been recommended for moderate to severely infected wounds based on potential pathogenic organisms mainly based on either gram-positive or gram-negative bacteria. In a review of 13 randomised control trials of different antibiotics comparison, there is no particular antibiotic that could be recommended for better clinical cure (Pratama et al., 2022).

Empirical treatment normally includes broad spectrum antibiotics in a severely infected wound such as Imipenem, Ciprofloxacin and 3rd and 4th generation cephalosporins (Armstrong & Lipsky, 2004; Lipsky, Peters, et al., 2012). In less severe infection, narrower oral antibiotics can be used (Armstrong & Lipsky, 2004). The empirical therapy was reported to be effective in 73%, with most cases treated with antibiotics covered for both gram-positive cocci and gram-negative rod (W. Wu et al., 2017). In another study, improvement following empirical therapy does not result in changes of antibiotics in 85% of the cases (Balakrishnan et al., 2014). In both studies, the choice of the empirical treatment, however, was dependant on the local prevalence data of infective pathogens and guidelines.

A combination antibiotic has been proposed to be more effective instead of a single agent to start the empirical treatment (Małecki et al., 2014). The antibiotics that were found to be more effective include a combination with aminoglycoside (Amikacin or Gentamicin) with either Piperacillin-tazobactam or Amoxicillin-clavulanate. Another study proposed Amikacin or Gentamicin to be used as empirical therapy in managing DFU (A. K. Singh et al., 2020). The proposed antibiotics from different studies are influenced by the local susceptibility data that can be different between centres. Furthermore, the susceptibility and prevalence of organisms can also change over time which then influences the local guideline. A combination of Amoxicillin-clavulanate with Trimethoprim-sulfamethoxazole was no longer found to be optimal after about three years, and a new combination of Amoxicillin-clavulanate with Ciprofloxacin was proposed (Carro et al., 2019). Despite the positive impact of empirical treatments, a recent study has reported a higher hospitalisation in patients with mild infection, involving skin and subcutaneous tissue that were treated empirically (Schmidt et al., 2023).

In many cases, empirical treatment is often followed with culture-based results for further management of the wound (Lipsky, 2016). Culture-based data will allow modification to antibiotic regimes to suit the susceptibility profiles of the isolated pathogens. Moreover, repeat culture will allow monitoring of the emerging of antibiotic resistance during treatment. Previously a suspected osteomyelitis cohort of DFU patients showed that about 77% of the wounds contained bacteria that were resistant to the empirical antibiotics (Tardáguila-García et al., 2019).

Apart from systemic antibiotics that are given either by oral or intravenous route, topical antimicrobials have also been used to treat DFU. Topical antimicrobial is not limited to the use of antibiotics but also includes antiseptics. Various antibiotics and antiseptics agents to treat chronic wounds were described here (Lipsky & Hoey, 2009). Unlike antibiotics which only work against bacteria, antiseptics such as Cetrimide, Iodine compounds and sodium hypochlorite are also active against fungi (Dumville et al., 2017). Microbial coverage can vary between compounds. A previous systematic review based on four randomised control trials comparing topical antimicrobials with systemic antibiotics could not be certain of the advantages of the topical treatment due to the limited data reported.

However, both treatments seem to give similar side effects to the subjects treated with either topical or systemic antibiotics (Dumville et al., 2017). On the other hand, there have been case reports and case series showing successful treatment with topical antibiotics, either alone or in combination with systemic antibiotics (Markakis et al., 2018). Lower concentrations represented by sub-MIC of systemic antibiotics in the tissue are common in DFU (Crowther et al., 2021). An i*n vitro* experiment demonstrated a 5-8 log reduction of bioburden when biofilms were treated with high concentration of Vancomycin and Gentamicin loaded Calcium-sulfate beads compared to when biofilms were exposed to antibiotic concentration similarly detected in the tissue (Crowther et al., 2021). The topical delivery of antibiotics to achieve high concentration of antibiotics is promising especially against biofilms.

With antibiotics treatment, biofilms do not show a reduction in bioburden when fungi are present (Crowther et al., 2021). Evidence on the antifungal treatment in DFU is still very limited. One study by Heald and colleagues highlights a positive response following antifungal treatment with Flucytosine, Fluconazole, Itraconazole and Terbinafine (Heald et al., 2001). However, the study does not explicitly indicate the dose and duration of the antifungal treatment for each individual patient. In patients with mixed infections of bacteria and fungi, not all the wounds healed following antifungal treatments with Fluconazole, Itraconazole, Caspofungin or Amphotericin B. Some wounds required amputation despite the antifungal treatment (Öztürk et al., 2019). The addition of 150 mg Fluconazole daily to the standard care regime has accelerated wound closure in cases where Fluconazole-susceptible fungi mostly *Candida* species were isolated from the deep tissue (Chellan et al., 2012).

1.6.2 Biofilm-based approach

Managing biofilms in chronic wounds involves physical intervention and the use of an antibiofilm strategy (Rhoads et al., 2008) following clear evidence of biofilms, which was observed in 60% of chronic wounds (James et al., 2008). Figure 1.5 demonstrates the biofilm-based wound care (BBWC) algorithm from an early study to manage biofilm in chronic wounds (R. D. Wolcott & Rhoads, 2008). Debridement is a first critical step to remove non-viable tissues leading to exposure of extracellular matrix with normal blood supply. Sharp debridement is

recommended as part of the standard care (Everett & Mathioudakis, 2018). However, previous studies have demonstrated the use of multiple ways of debridement procedures to remove the dead tissues including mechanical and non-mechanical ways (Dayya et al., 2022; Ning et al., 2023). Non-mechanical debridement includes autolytic methods, such as with hydrogel and enzymatic debridement by using enzyme such as collagenase (Ning et al., 2023). The application of hydrogel has resulted in an increased wound healing rate in DFU when compared to gauze (Edwards & Stapley, 2010). It works by providing a moist wound environment, however, there is a concern regarding the use of hydrogel on dry gangrene that could potentially transform into wet gangrene (Hilton et al., 2004). Based on the network meta-analysis of randomised clinical trials, enzymatic debridement was found to be most effective in improving the healing rate of DFU (Ning et al., 2023). However, due to the low quality of the studies been analysed, further research is warranted to confirm its benefit. Another method which uses maggots may potentially be effective at removing the dead tissues, however, more evidence is needed for routine practice (Tian et al., 2013).

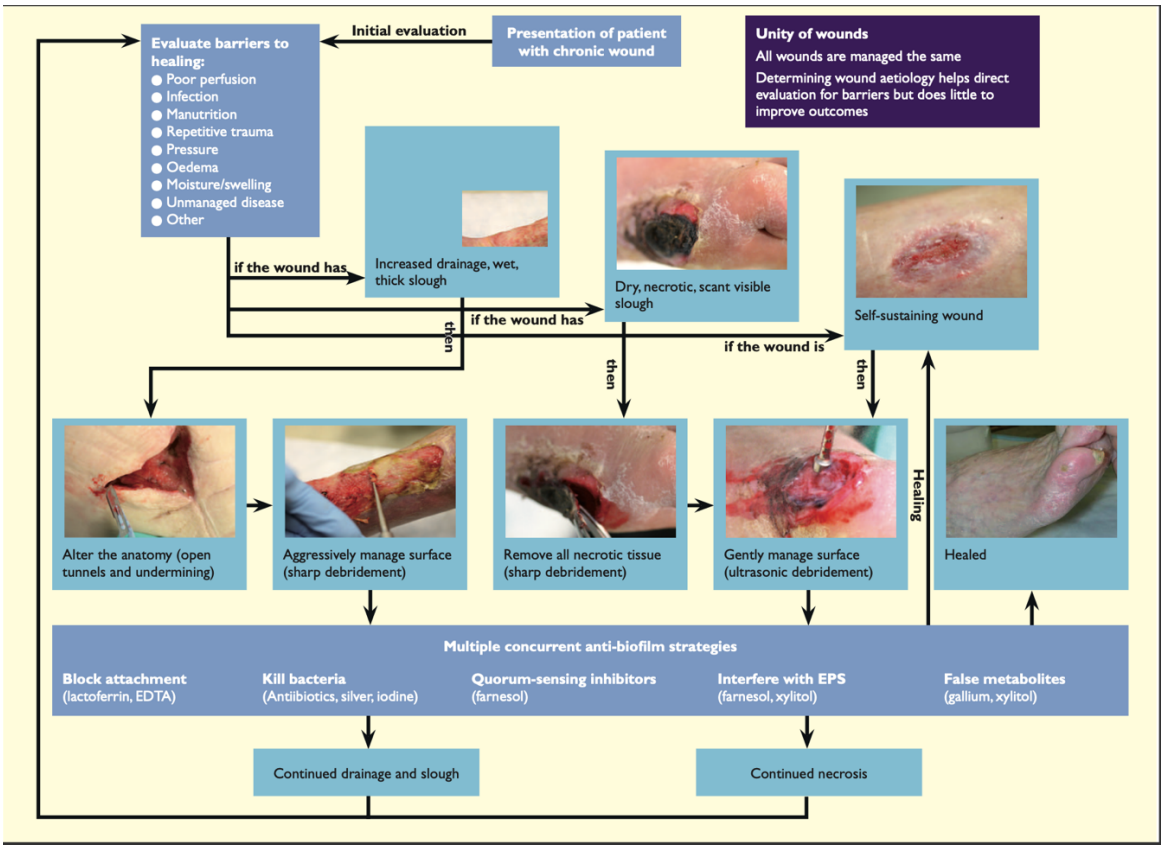


Figure 1.5 Biofilm-based wound care algorithm. (R. D Wolcott and Rhoads, 2008).

To date, any methods of debridement is not recommended by the IWGDF 2023 guideline except by sharp debridement (P. Chen et al., 2024). These includes autolytic, biosurgical, hydrosurgical, chemical or laser methods. However, in certain cases, enzymatic debridement can be performed when resource is limited but is not recommended for routine use over sharp debridement (P. Chen et al., 2024). Despite the physical removal of biofilms through repeated debridement, there was a strong agreement that is not enough to prevent the regrowth of biofilms and therefore further intervention is required including the use of topical antiseptics (Schultz et al., 2017).

The next step as part of the BBWC is to inhibit, and also prevent, biofilm formation. These can be achieved by utilising multiple antibiofilm compounds with different modes of action. These include compounds that inhibit attachment and quorum-sensing, as well as compounds that will kill the bacteria such as antibiotics. When standard care was combined with a compound that destroyed the EPS, the percentage of wounds healed increased to 93% compared to only 53% with just standard care (R. Wolcott, 2015a).

1.6.3 Novel therapy

A perpetual attempt to tackle the ongoing burden of DFU can be seen from past studies that explore alternative and new methods to eliminate the infection. While management of the DFU is multifaceted, the review here focuses on the novel method from a microbiology perspective.

One of the reasons for the search for novel antibiotics is due to the emergence of resistance and the failure of the current antibiotics to treat biofilms (Pouget et al., 2021). This could be attributed to the poor tissue penetrations with systemic antibiotics as reflected by the low concentration of the antibiotics in the infected wounds (Crowther et al., 2021). Therefore, studies on new antimicrobial strategies involve the development of novel compounds and improvement of drug delivery to optimise the bioavailability of the antibiotics at the targeted site.

Dalbavancin is one of the novel antibiotics belonging to Lipoglycopeptide class that has shown promising activity against mostly gram-positive bacteria including multi-resistant organisms such as MRSA (Mougakou et al., 2023). It has also been

recognised with a very long half-life. Treatment of DFI with osteomyelitis with this antibiotic against gram-positive bacteria including *S. aureus* and *C. striatum* has shown a good potential in treating diabetic foot infection (Navarro-Jiménez et al., 2022). With its good activity against *S. aureus*, *in vitro* study has shown anti-biofilm activity against biofilms formed by MRSA isolated from the infected bone (V. Silva et al., 2021). A combination treatment with ficin, a biofilm detaching compound has enhanced the activity of Dalbavancin against MRSA (Žiemytė et al., 2020).

Development of a novel drug-delivery strategy with calcium sulfate beads loaded with Vancomycin and Gentamicin has substantially increased the reduction of bacteria ranging from 2 – 8 log CFU/mL in polymicrobial biofilms model consisting of organisms isolated from wounds (Crowther et al., 2021). A similar study with Tobramycin or Gentamicin-loaded calcium-sulfate beads was able to completely eliminate *P. aeruginosa* biofilms (L. et al., 2016). Another strategy of drug-delivery is the application of bioprinting technology that creates a 3D scaffold loaded with Levofloxacin that shows an inhibitory effect against *E. coli* and *S. aureus* (Glover et al., 2023). The scaffold can sustain a continuous release of antibiotics for up to four weeks. Apart from antibiotics, natural compounds such as essential oil can also be incorporated into the scaffold. However, these studies are still at a very early stage before can be applied for clinical use.

Despite novel approaches, when fungi are present, antibiotics do not work (Crowther et al., 2021). A novel mechanism based on the production of reactive oxygen species produced by cold atmospheric plasma (CAP) has shown promising effects on DFU (Mirpour et al., 2020). *In vitro* study of biofilm treatments consisting of triadic species that are commonly found in chronic wounds including *Candida* has shown significant reduction when exposed to five minutes treatment of CAP (Baz et al., 2023). In the same study, treatment with CAP also reduced the viability of *C. auris* biofilms.

1.7 Aims and hypothesis

Previous studies reviewed in this chapter have highlighted the important role of microbiome in driving the chronicity of the wound. Despite antibiotic treatments, wounds remain unhealed. There is a lack of understanding of the involvement of fungi in influencing the dynamic of the wound microbiome as the wounds progress.

It is hypothesized that wound microbiomes are patient-specific and involve interplay between bacteria and fungi within the biofilms that will determine the outcome of clinical treatments. Therefore, this study aims to characterise the polymicrobial community of DFU and model the impact of antimicrobial treatments on this complex wound community. Specifically, the work presented in this thesis aims to :

- Identify bacteria and fungi isolated from standard culture
- Characterise the 16S microbiome using Oxford Nanopore sequencing platform
- Demonstrate the dynamic of wound microbiome in selected patients
- Identify fungi from the wounds using enhanced mycology culture
- Model the impact of antimicrobial therapy using real-world biofilms model

2 Standard microbiology culture

2.1 Introduction

As empirical treatment is usually started before the culture result is available, evidence and prior knowledge on the prevalence of causative pathogen is highly important to facilitate the appropriate selection of antibiotics. Empirical guideline for treatment of infected DFU includes recommendation of do's and dont's when selecting antibiotics. The proposed empirical guidelines by IWGDF/IDSA are to treat the wounds that are mild, moderate or severe that are likely caused by pathogens grouped based on the gram stain. This includes coverage for only gram-positive bacteria including, *S. aureus* and beta-hemolytic *Streptococcus* in mild-infected DFU, and only target against *P. aeruginosa* with previous history of isolation from the affected site (Senneville et al., 2024). Certain resistance species such as MRSA and ESBL-producing organisms are also included as potential pathogen to be treated empirically.

The IWGDF/IDSA guidelines recommend to initially identify the pathogen using microbiology culture instead of the molecular method in infected DFU (Senneville et al., 2024). This is because, standard culture will allow identification and antibiotic susceptibility testing to be performed to guide selection for appropriate antibiotics. Moreover, molecular method is unable to differentiate between live and dead organisms in the samples that might lead to misuse of antibiotics. The recovery of viable organisms from microbiology culture is suggestive of the ongoing proliferation of the causative microbes associated with the non-healing wound or indicative of resilience to treatment. A prior study on patients with mild DFU infection has shown lower incidence of hospitalization in patient guided by culture result than empirical treatment (Schmidt et al., 2023). It is therefore important to be aware of the isolated pathogen from the culture to make an informed decision while managing the wound.

In a multicentre trial based in the United State, a diverse bacterial species has been reported from culture of moderate to severe wounds as classified according to the definition compatible with the Infectious Disease Society of America wound classification system (Citron et al., 2007). Although this system is widely used, however, the criteria is rather complex (Monteiro-Soares et al., 2019). Surprisingly, despite a large sample size of 454 samples collected before any antibiotic treatment, only bacteria and no fungi were reported in either pure or

mixed growth (Citron et al., 2007). On the other hand, Sanniyasi and colleagues have only reported fungi from wounds of certain grade with 42% of the wounds are from Wagner's grade III (Sanniyasi et al., 2015). There is a lack of study that simultaneously covers bacteria and fungi using one classification system. Therefore, stratifying the diversity of organisms isolated from culture according to different grades and stages will improve in proper selection for the appropriate antibiotics.

2.2 Aims

The aim of this chapter was to identify a spectrum of organisms isolated from standard microbiology culture and analyse them according to their respective wound grades and stages based on the University of Texas wound classification system.

2.3 Materials and methods

2.3.1 Study participants

A total of 127 patients with diabetic foot ulcer were involved in this study with ethical approval obtained from HRA and Health and Care Research Wales (HCRW)(IRAS293291). Patients' assessment and demographic data collection were performed by the clinical team at Royal Lancaster Infirmary (RLI) who attended to the patients and were recorded into a standardised sheet. Demographics data involved information on their age and sex and, every patient was identified numerically to ensure their anonymity.

2.3.2 Sample collection and processing

Swabs were collected by the attending podiatrist during the clinic appointment. Assessment on the location and the condition of the wound were recorded based on the University of Texas wound classification system (Lavery et al., 1996). Briefly, wounds were categorised according to their grade 0, I, II and III that corresponds to the wound depth and also their stage A, B, C and D that indicates the presence of infection or ischemia. Standard microbiology culture was performed by biomedical scientists at the microbiology laboratory of RLI according to the UK Standards of Microbiology Investigations (England, 2018). The procedure involved the use of standard microbiology media that allow for the isolation of both bacteria and fungi , incubated at 35-37℃ and 28-30℃, respectively. In addition, wounds were also incubated in anaerobic incubation for the isolation of anaerobes. Results from the standard culture were collected by Dr Anna Cassey.

2.4 Results

2.4.1 Patient demographics

A total of 127 patients were enrolled in this study. Table 2.1 shows their basic demographic data . Majority of the patients were male (78%) compared to female (22%). The median age of the total patients irrespective of the gender was 67 years old.

Table 2.1 Patient demographic data.

Characteristics	N = 127
Age	67 (56, 75)
Sex	
Male	99 (78 %)
Female	28 (22 %)
Median (IQR), n (%)	

2.4.2 Wound characteristics

All the wounds were classified according to the University of Texas classification system. Figure 2.1 shows the number of samples corresponding to the grades and stages of the wound involved in this study. A total of 349 samples were assessed in this study. However, 13 samples were unavailable to process for standard culture. The wound grade represents the extent of tissue damage ranging from mild, the absence of skin break (Grade 0) to the severe one, penetrating bone (Grade III). The wounds swabbed were mostly superficial, grade I (57%) followed by grade III (25.8%), grade II (16.3%) and grade 0 with less than 1%. On the other hand, stages represents the presence of infection or ischemia. Infected wounds, stage B (54.7%) were predominant in this study compared to uninfected wound, Stage A (31.2%). The most common combination of grade and stage is BI (infected superficial wound) with 26.6% followed by uninfected superficial wounds AI with 25.5%.

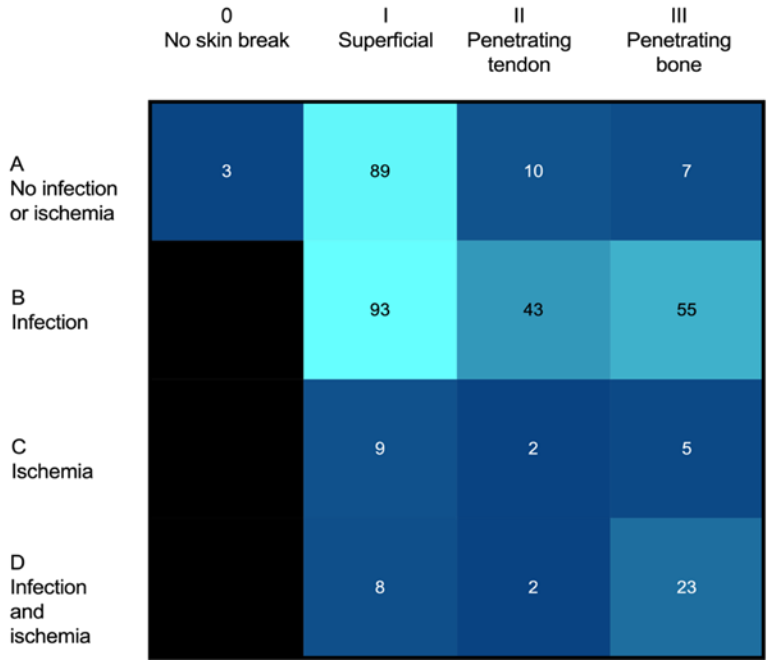


Figure 2.1 Wound grades and stages according to the University of Texas classification system. Each column shows the grade of the wound from 0 to III respective to the depth of the wound while each row shows the stage of the wound from A to D that indicate the presence or absence of infection and ischemia. Numbers in each square shows the number of wound swabs processed in this study.

2.4.3 Relative occurrence of isolated organisms in all samples

A total of 306 culture data were reported with growth, 13 samples with no culture result and 30 samples reported with no growth or no significant growth. Majority of the wounds were reported to consist of mix growth rather than single species (Figure 2.2). Higher percentage of polymicrobial growth (85.6%) was observed compared to monoculture (14.4%). From a closer look into the culture data, monoculture predominantly consisted of *S. aureus* followed by *Pseudomonas* at 70.5% and 13.6%, respectively. On the other hand, 61.5% of the polymicrobial samples consisted of gram-negative bacteria compared to only 38.5% gram positive bacteria. Co-isolation with *Candida* and anaerobes were found to be similar at 11.5% and 13%, respectively.

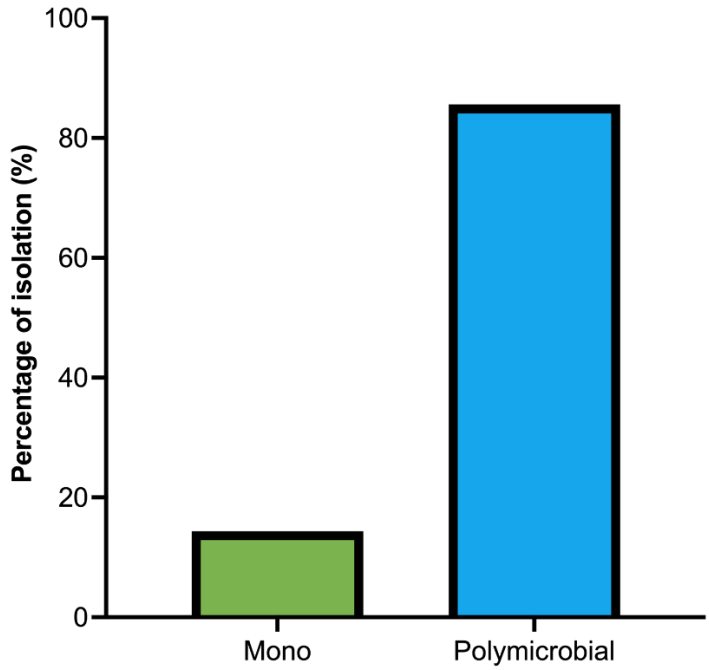


Figure 2.2 Isolation of mono versus polymicrobial growth. Bar graphs show the percentage of culture identified as monoculture and polymicrobial out of 306 samples with growth. Mono and polymicrobial were found in 44 (14.4%) and 262 (85.6%) samples, respectively.

Next, the relative occurrence of the different organisms among the samples were analysed and presented in Figure 2.3 Data shown here represent the percentage of the organisms from all the samples with growth (number of a particular bacteria divided by the total number of isolates). Not all the organisms isolated were reported to genera and species level, but were based on commonly reportable terms including coliforms, enterics and mixed skin. The most frequently isolated organisms were mixed skin (25.6%) followed by enterics (16.9%). *S. aureus* was the most frequently identified species of gram-positive bacteria at 16.7%. The *Pseudomonas* species includes *P. oleovorans* (one isolate) and other isolates that were not identified to the species level. *P. aeruginosa* was individually plotted at 1% as this bacterium is commonly reported as an important pathogen in wound (Garousi et al., 2023). *Candida* species and anaerobes were similarly isolated from the wound at 6.1% and 6.9%, respectively. Other less frequent organisms isolated in this study include *Streptococcus* species, *Enterococcus* species, *Corynebacterium striatum* and *Staphylococcus* species.

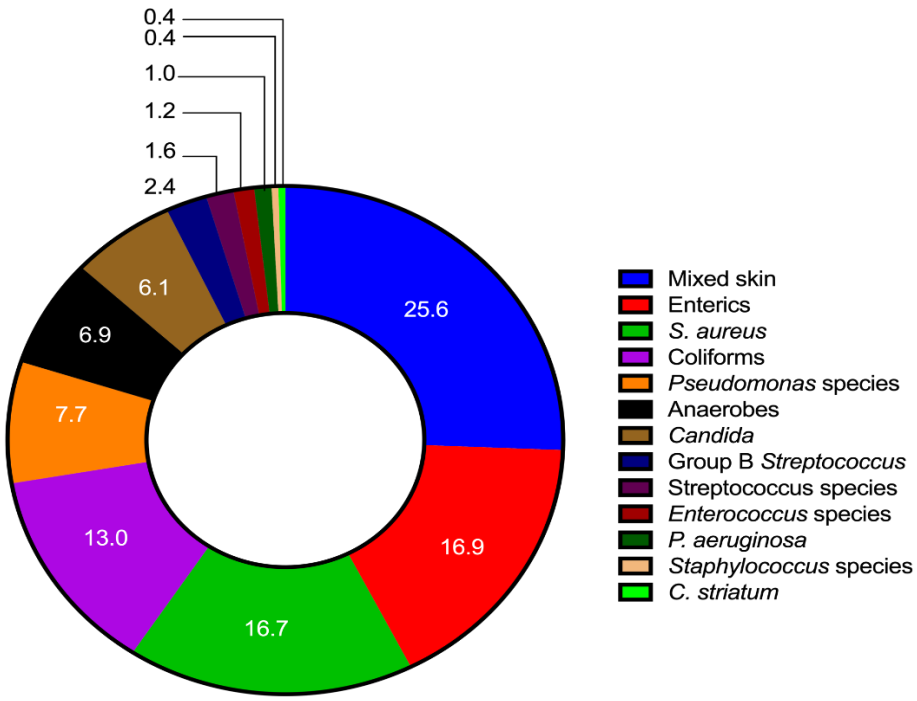


Figure 2.3 Organisms isolated from all samples. A pie chart shows a percentage of organisms isolated from the wound samples. Data represent the percentage of relative isolation of the organisms from all grades and stages that were obtained from 306 samples with growth.

2.4.4 Relative occurrence of isolated organism according to wound stages

Table 2.2 shows the distribution of every organism identified from all the four stages of the wounds. There were limited identifications of the species that can be observed based on the standard culture as bacteria were reported according to the functional and ecological grouping such as enterics and anaerobes. From the table, the prevalence of every organism isolated can be compared between the stages. It is important to note that the total number of samples cultured for each stage was not comparable as fewer samples were collected for stage C and D. Therefore, it is more practical to make a comparison between non-infected versus infected (A versus B) and ischemic versus infected-ischemic wounds (C versus D). Based on the aerotolerance of the isolates, wounds predominantly consisted of facultative bacteria.

Table 2.2 Organisms isolated according to wound stages. Table shows the organisms of gram-positive, gram-negative, fungi and anaerobes isolated from standard culture of varying stages according to the University of Texas wound classification system.

Organisms	Wound stages				Aerotolerance
	A (n=106)	B (n=185)	C (n=14)	D (n=31)	
Gram positive					
S. aureus	23	37	3	10	Facultative
MRSA	4	3	2		Facultative
S. lugdunensis	1				Facultative
S. simulans	1				Facultative
Group B *Streptococcus*	2	9	1		Facultative
Streptococcus Group C		1			Facultative
Streptococcus Group G	2	3		1	Facultative
S. milleri		1			Facultative
Enterococcus species	1	4			Facultative
E. faecalis	1				Facultative
C. striatum		2			Facultative
Gram negative					
Enterics	17	52	1	10	Facultative
E. cloacae	1				Facultative
Proteus species		2			Facultative
Coliforms	23	30	4	7	Facultative
Pseudomonas species	8	25		4	Aerobic
P. aeruginosa	1	3		1	Aerobic
P. oleovorans	1				Aerobic
Fungi					
Candida species	5	8	4	3	Facultative
C. albicans	4	6			Facultative

Others					
Anaerobes	8	20	3	3	Anaerobic
Mixed skin	35	76	5	10	N/A
No growth/ No significant growth	17	12	1		N/A

Non-infected versus infected wounds (A vs B)

Table 2.3 shows the prevalence of the common organisms isolated from non-infected and infected wounds. Among gram positive bacteria, *S. aureus* was similarly observed between non-infected and infected wounds. On the other hand, *Streptococcus* was more frequently isolated in infected wound. However, there was no statistical difference observed for both organisms. For gram negative bacteria, enterics and coliforms were found in both stages, with enterics showing statistically significant differences between the stages. Furthermore, anaerobes were frequently reported in infected than non-infected wounds. Surprisingly, mixed skin bacteria were more frequently isolated in infected wounds, though no statistically significant differences were observed. The isolation of fungi was similar for both stages.

Table 2.3 Comparison of common organisms from non-infected versus infected wounds. Data presented in this table compared the number of organisms isolated from stage A (non-infected) and stage B (infected) wounds. Certain organisms were grouped together as indicated in the table. Fisher's Exact test was used to compare between the two stages with $p < 0.05$ was considered as statistically significant. * indicates statistically significant difference.

Organisms	Stage A (n=106)	Stage B (n=185)	p - value
S. aureus (including MRSA)	27 (25.5%)	40 (21.6%)	0.4717
Streptococcus (including Group B *Strep*, *Strep*. Group C, G and *S. milleri*)	4 (3.8%)	14 (7.6%)	0.3113

Enterics (including *E. cloacae*, *Proteus* sp.)	18 (17%)	54(29.2%)	0.0237*
Coliforms	23 (21.7%)	30 (16.2%)	0.2706
Pseudomonas (including *P. aeruginosa*, *P. oleovorans* and *Pseudomonas sp.*)	10 (9.4%)	28 (15.1%)	0.2063
Anaerobes	8 (7.5%)	20 (10.8%)	0.4147
Mixed skin	35 (33%)	76 (41.1%)	0.2098
Candida (including *C. albicans* and *Candida sp.*)	9 (8.5%)	14 (7.6%)	0.8230

Ischemic versus infected-ischemic wounds (C vs D)

To compare if the presence of infection in ischemic wounds constitute differences in organism, wounds of stage C and D were compared. The isolation of the most common organisms was compared between stage C and D in Table 2.4. From the table, none of the organisms isolated show statistically significant differences between the two stages. Percentage of isolation was similar for most organisms of either stage, except for enterics which had higher percentage of isolation in stage D (32.3%) compared to stage C (7.1%).

Table 2.4 Comparison of common organisms from ischemic and infected-ischemic wounds. Data presented in this table compared the number of organisms isolated from stage C (infected) and stage D (infected-ischemic) wounds. Certain organisms were grouped together as indicated in the table. Fisher's Exact test was used to compare between the two stages with $p < 0.05$ was considered as statistically significant.

Organisms	Stage C (n=14)	Stage D (n=31)	p - value
S. aureus (including MRSA)	5 (35.7%)	10 (32.3%)	>0.9

Enterics	1 (7.1%)	10 (32.3%)	0.1318
Coliforms	4 (28.6%)	7 (22.6%)	0.7171
Mixed skin	5 (35.7%)	10 (32.3%)	>0.9
Candida (including *C. albicans* and *Candida sp.*)	4 (28.6%)	3 (9.7%)	0.1797

In order to have a better understanding on composition of organisms within each stage, relative occurrence of the organism isolated was plotted in Figure 2.4. In non-infected (stage A), mixed skin constituted the most predominant isolate followed by *S. aureus*, coliforms and enterics in descending order. Anaerobes and *Candida* were found to be in similar proportion. Similar composition was observed in infected (stage B) wound, however, enterics was slightly higher than *S. aureus*. On the other hand, ischemic wound (stage C) was predominated by *S. aureus*. Interestingly, *Candida* accounted for 17.04% relative to other organisms isolated within the stage, higher than enterics that were predominant in stage A and B. Finally, in infected-ischemic wound (stage D), *S. aureus*, mixed skin and enterics were found to be the predominant organisms at the same proportion. Anaerobes and *Candida* were also presence at the same proportion of 6.1%.

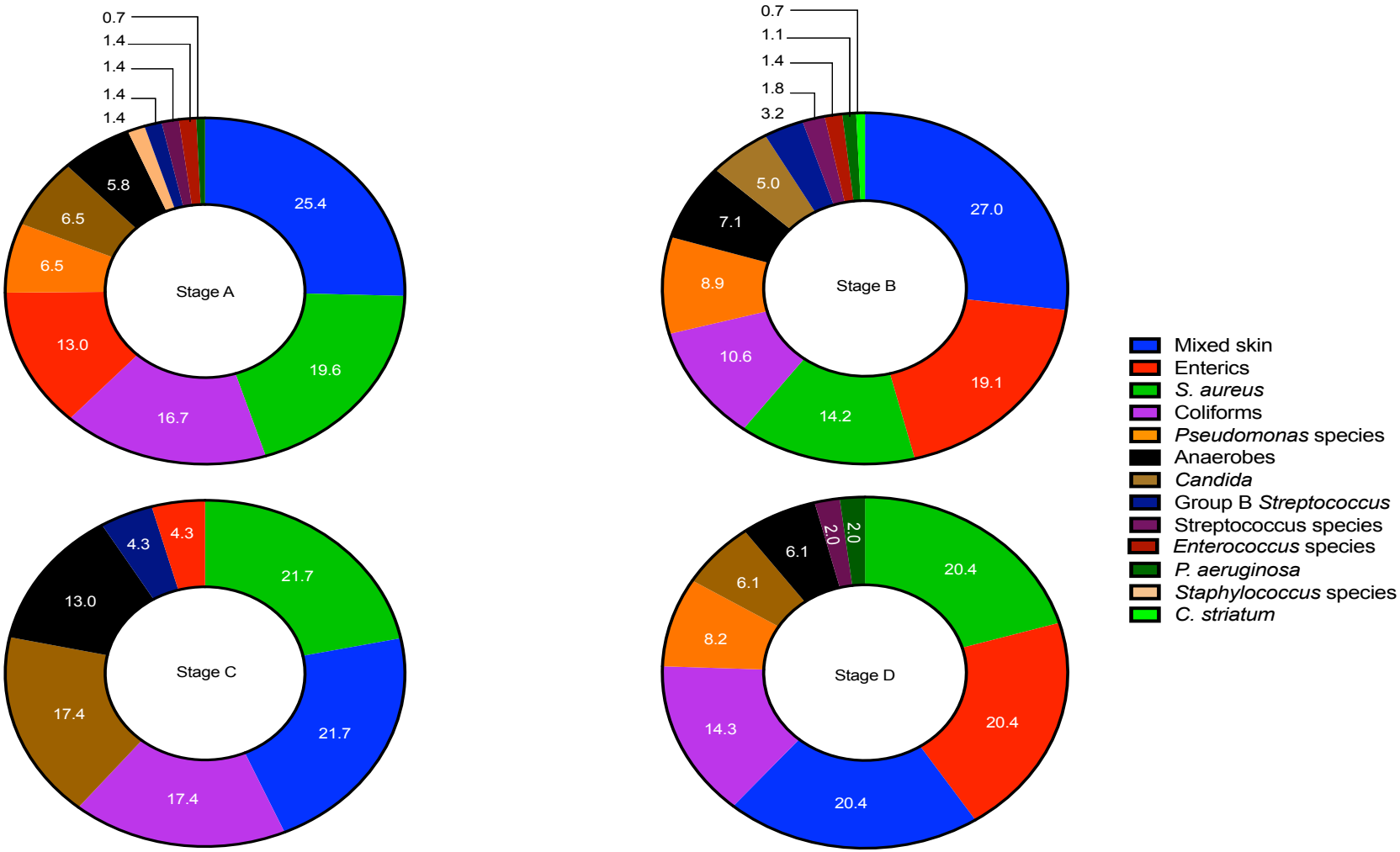


Figure 2.4 Organisms isolated according to wound stages. Pie charts illustrate relative proportion of organism isolated from different stages of wound according to University of Texas classification system.

2.4.5 Relative occurrence of isolated organisms by wound grades

The distribution of every organism isolated according to the depth of the wound was presented in Table 2.5. To compare the prevalence of the most common organism between the grades, only grade I, II and III were analysed as grade 0 has a very small sample size.

Table 2.5 Organisms isolated according to wound grades. Table shows the organisms of gram-positive, gram-negative, fungi and anaerobes isolated from standard culture of varying grade according to University of Texas wound classification system.

Organisms	Wound grades				Aerotolerant
	0 (n=2)	I (n=195)	II (n=55)	III (n=84)	
Gram positive					
S. aureus	1	50	8	14	Facultative
MRSA		8	1		Facultative
S. lugdunensis		1			Facultative
S. simulans		1			Facultative
Group B *Streptococcus*	1	6	3	2	Facultative
Streptococcus Group C		1			Facultative
Streptococcus Group G		5		1	Facultative
S. milleri		1			Facultative
Enterococcus species		4	1		Facultative
E. faecalis		1			Facultative
C. striatum				2	Facultative
Gram negative					
Enterics	1	43	13	23	Facultative
E. cloacae		1			Facultative
Proteus species			2		Facultative
Coliforms		38	11	15	Facultative
Pseudomonas species		21	6	10	Aerobic

P. aeruginosa	3		2	Aerobic
P. oleovorans	1			Aerobic
Fungi				
Candida species	12	4	4	Facultative
C. albicans	7	2	1	Facultative
Others				
Anaerobes	17	5	12	Anaerobic
Mixed skin	67	22	37	N/A
No growth/ No significant growth	21	5	4	N/A

From Table 2.6, the isolation of *S. aureus* shows statistically significance difference between the grades (p= 0.0224) as measured by Fisher's Exact test. However, multiple comparisons with Bonferonni correction does not indicate a statistical significance difference. However, *S. aureus* was most commonly identified from a superficial (grade I) wound. As the wound involved a deeper tissue, there was an increase in anaerobes, however, this does not show a statistical significance difference. An increasing trend was also observed for mixed skin as the wound increased in grade. Other organisms show a comparable prevalence across all grades.

Table 2.6 Comparison of common organisms from grade I, II and III. Data presented in this table compared the number of organisms isolated from the three grades. Certain organisms were grouped together as indicated in the table. Fisher's Exact test was used to compare between the two stages with p< 0.05 was considered as statistically significant. * indicates statistically significance.

Organisms	Grade I (n=195)	Grade II (n=55)	Grade III (n=84)	p - value
S. aureus (including MRSA)	58 (29.7%)	9 (16.4%)	14 (16.7%)	0.0224*
Streptococcus (including Group B *Strep*, *Strep*. Group C, G and *S. milleri*)	13 (6.7%)	3 (5.5%)	3 (3.6%)	0.6372

Enterics (including *E. cloacae*, *Proteus* sp.)	44 (22.6%)	15 (27.3%)	23 (27.4%)	0.5819
Coliforms	38 (19.5%)	11 (20%)	15 (17.9%)	0.9431
Pseudomonas (including *P. aeruginosa*, *P. oleovorans* and *Pseudomonas sp.*)	25 (12.8%)	6 (10.9%)	12 (14.3%)	0.8285
Anaerobes	17 (8.7%)	5 (9.1%)	12 (14.3%)	0.3569
Mixed skin	67 (34.4%)	22 (40%)	37 (44%)	0.2913
Candida (including *C. albicans* and *Candida sp.*)	19 (9.7%)	6 (10.9%)	5 (6%)	0.4964

Relative occurrence of organisms isolated according to wound grade is presented in Figure 2.5. Grade 0 that represent a pre/post ulcer without any skin break only involved two samples with similar occurrence between enterics, Group B *Strep.* and *S. aureus*. In superficial wound (grade I), mixed skin was slightly at higher occurrence than *S. aureus* with 23.3% and 20.1%, respectively. *Candida* species accounted for 6.6% and was higher than bacteria including anaerobes, *Streptococcus* species and *Enterococcus* species. As ulcer extend to a deeper tissue penetrating tendon (grade II), mixed skin remains the most predominant organism at 28.2% followed by enterics and coliforms at 19.2% and 14.1%, respectively. *S. aureus*, a facultative anaerobe represents at 11.5%, slightly higher than *Candida* and *Pseudomonas* which were both at 7.7%. The presence of anaerobes accounted for 6.4% while others including Group B *Strep.* and *Enterococcus* was each below 5%. Finally, in wound that involved the bone (grade III), mixed skin constituted for 30.1% followed by enterics at 18.7%. The proportion of anaerobes at 9.8% was higher than others including *Pseudomonas*, *Candida*, *C. striatum* and *Streptococcus*.

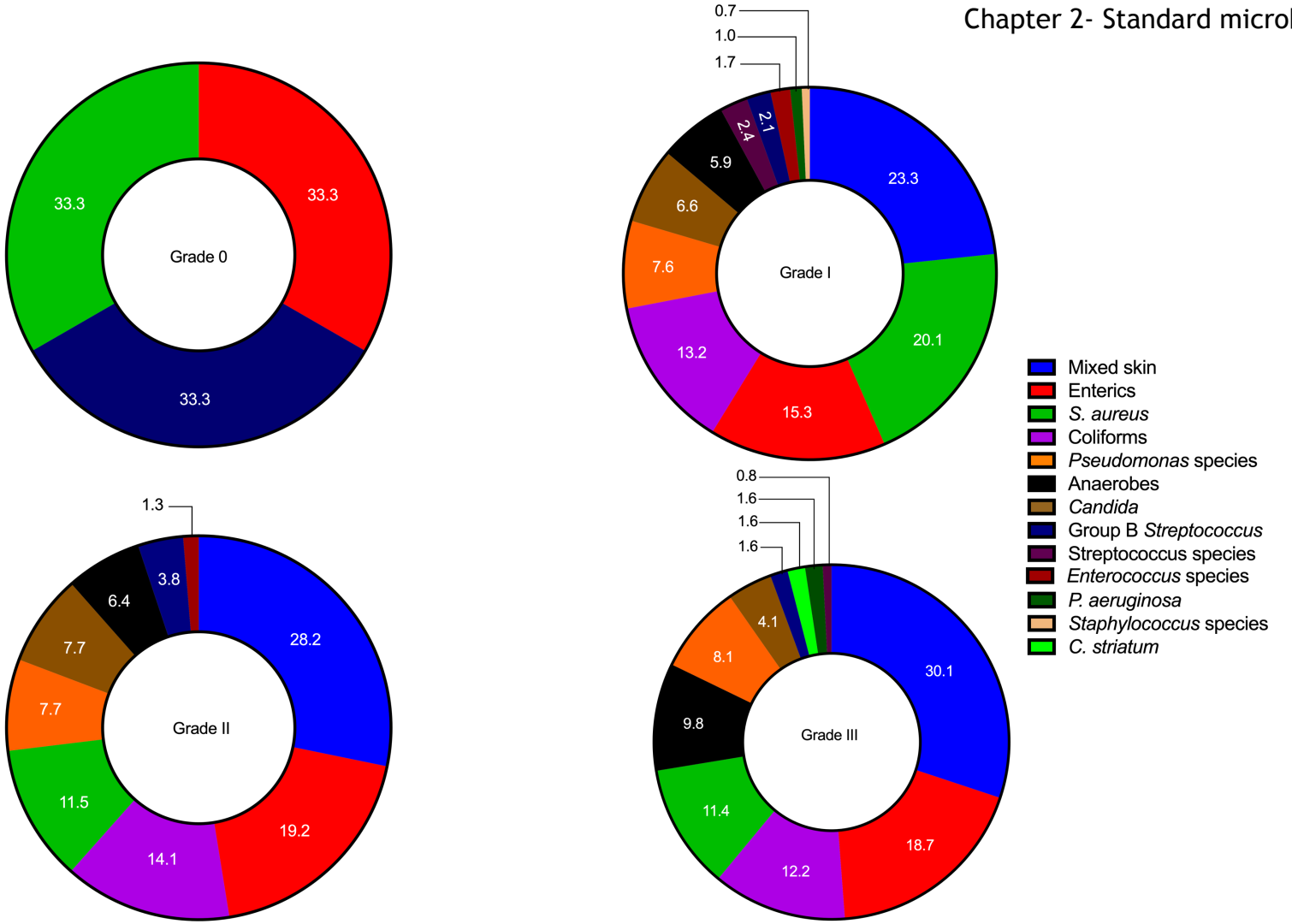


Figure 2.5 Organisms isolated according to wound grades. Pie charts illustrate a percentage of organisms isolated from different grades of wound according to University of Texas classification system. Percentage represents the relative occurrence of the organism within each grade.

2.5 Discussions

The management of infected DFU often begins with empirical antibiotic treatment against the most common pathogenic bacteria primarily gram-positive bacteria such as *Staphylococcus* and *Streptococcus* species or based on the availability of local prevalence data (Matheson et al., 2021). Notwithstanding the benefit of pre-emptive treatment, the evidence of the causative organism must be verified by the isolation of the pathogenic organism from the wound (Lipsky et al., 2004). This is important in choosing an appropriate treatment in cases of poor response to the empirical treatment. Wounds are often classified based on the severity and empirical treatments against potential pathogens are primarily based on gram-stain (Senneville et al., 2024). According to the University of Texas wound classification system, wounds are presented with different grades and stages, therefore it is crucial to address variety of organisms that are responsible in each category to have a well-informed decision on what to look for while diagnosing the cultured samples. It was evidenced from the data demonstrated in this chapter that wounds were primarily polymicrobial and comprised of mainly bacteria of skin and gut origin.

Indeed, previous studies have reported the isolation of more than one organism from diabetic wounds (Abdulrazak et al., 2005; Citron et al., 2007; Hitam et al., 2019; Tascini et al., 2011). Similarly, the polymicrobial existence of diabetic wound was also apparent in this present study with 85.6% samples were mixed growth. It has been shown that polymicrobial interaction is associated with increased in virulence and in the context of chronic wound this often manifested as biofilms formation(Dalton et al., 2011; Keogh et al., 2024). Therefore, progressive non-healing wound that were not responding to antibiotic treatment should be indicative of the formation of polymicrobial biofilms that may be benefitted from the biofilms based wound care treatment (Schultz et al., 2017). In a multicentre study consisted of over 400 culture-positive samples, isolation of four or more organisms accounted for about 40% of these (Citron et al., 2007) with other study reported an average of about two organisms (Abdulrazak et al., 2005). However, it was uncertain to determine the number of species that were co-isolated in this study as bacteria were identified based on the ecological niche such as enterics or mixed skin which can include many species. Perhaps a diverse

range of species could have been identified if the isolates were further differentiated into a lower taxonomic level.

Apart from bacteria, isolation of *Candida* species was also observed in this study with all the *Candida* isolates were found to co-exist with bacteria. This in line with other microbiology culture studies that have also reported the isolation of fungi mainly *Candida* at varying frequency (Abdulrazak et al., 2005; Goh et al., 2020). Their co-isolation with bacteria strengthens the evidence of synergistic interaction is not only limited to between bacteria but also across the kingdoms. These findings have implications for treatment as current guidelines does not address the treatment when fungi are present (Senneville et al., 2024). In addition, detection of fungi was often limited to the standard growth rate of common bacteria that often grow faster than fungi. Thus, enhancing the growth condition to accommodate specifically for fungi may potentially improve their detection in chronic wound. On the other hand, samples with single growth often consisted of *S. aureus* followed by *Pseudomonas* species . Similarly, the single isolation of these organisms in culture has also been frequently observed in other studies (Abdulrazak et al., 2005; Tascini et al., 2011). The interaction between *P. aeruginosa* and *S. aureus* has been an ongoing debate. While synergistic interactions have been reported in *in vitro* models, *in situ* detection of these two organisms inside the wound has shown that they were separated and occupying at different depth of the wound (Thaarup et al., 2022). From this same study, *P. aeruginosa* has been shown to be found deeper in the tissue compared to *S. aureus* and their co-isolation therefore is possible when proper sampling is performed. However, the precise interaction between these organisms cannot be confirmed in this study but perhaps sensitive detection by molecular method would be able to elucidate their co-existence.

When looking at the proportion of the organisms across all the samples, the predominance of mixed skin, enterics and coliforms has suggested the sources of most of the bacteria were the skin and gut. In a study that recovered about 30 000 isolates with an average of 698 isolates per sample has shown that 81.4% of the identified organisms have been isolated from stool (Jneid et al., 2018). The high prevalence of enterobacteriacea has been similarly observed since over 30 decades ago (Wheat et al., 1986). Due to location close to anorectal region, normal skin of below the waist has also been commonly found with bacteria from

the gut (Ki & Rotstein, 2008). Despite their high prevalence, enterobacteriacea were found to be less virulent in diabetic wound compared to *S. aureus* and *P. aeruginosa* and was thought to be indicator for better patient outcome (Mihai et al., 2014). By looking at the proportion in every stage in this study, their prevalence remains high compared to other organisms isolated. Whether or not their presence indicate infection cannot be determine in this study however, their presence must have played an important role in wound healing.

Comparison between wound stages does not indicate the differences in isolating a particular organism. Most studies of infected wounds have reported a high prevalence of *S. aureus* (Macdonald et al., 2021). While *S. aureus* is commonly seen from infected wound in this study, however, non-infected wound was also found to be similar. Although it is common to find *S. aureus* to colonise the skin, study has shown that persistence of *S. aureus* in infected wound is rare over 30 weeks period based on the persistence of clonal strain hence suggesting the reinfection rather than the same infection (Lavigne et al., 2021). Further study into the strain difference of *S. aureus* has shown that, some strains were found only in unhealed wound that contains multiple resistance gene and staphylococcal enterotoxin gene while some strains were presence in all healing outcomes (L. R. Kalan et al., 2019). Therefore, isolation of *S. aureus* does not necessarily indicate infection and must be clinically assessed properly as not to overlook the actual infective organisms.

Isolation of anaerobes were always accompanied by aerobic or facultative anaerobic organisms in this study. In fact, most studies have reported the same observations (Goh et al., 2020; K. Smith et al., 2016). However, Anyim et. al has found that anaerobes were more predominant than aerobe and in fact has found that 7% of the anaerobes were isolated in monoculture (Anyim et al., 2019). An average of four anaerobic species were isolated and identified from each sample with *Peptostreptococcus* was the most common (Ng et al., 2008). However, it was unable to compare the prevalence of the anaerobic species in this study as the isolates were not processed for identification. Furthermore, the finding also corroborates the study by Dowd et al. who suggest the functional equivalent pathogroup were all containing anaerobic organisms (Dowd, Wolcott, et al., 2008). A 6.9% of the anaerobes were identified in this study out of all the identified isolates. Similar average of 7.45% was reported from analysis of multiple studies

(Villa et al., 2024). However, the prevalence of anaerobes reported in this present study does not account for individual species but rather than the group of bacteria. Hence, this average is anticipated to be lower if the identification of individual isolates were performed. This implies there is still a challenge in culturing the anaerobic organisms. Furthermore, the isolation of anaerobes was found to increase as the wound depth increased. This has been similarly seen in other studies that show an increasing isolation of anaerobes in a deeper wound (Sasikumar et al., 2018). According to the IWGDF/IDSA guideline (Senneville et al., 2024), empirical treatment against anaerobes was proposed for severe and ischemic wound, however data from this present study showed that non-ischemic wound is similar to infected ischemic wound in the presence of anaerobes. Therefore, anaerobes should also be considered regardless the presence ischemia.

Taken together, standard microbiology culture has not distinguished a particular group of organisms that can be associated with severity or the wound depth. Nevertheless, this study has identified group of bacteria stratified according to the different grades and stages of the wound that underscore the major source of the organisms that could potentially thrive in DFU. Since the study was limited to accurate identification of the species, it was not possible to delineate precisely the species interaction that might either lead to the improvement or exacerbation of the wound as DFU was found to be mostly polymicrobial. Despite so, data presented here does align with recommendation of the empirical treatment for DFU (Senneville et al., 2024). Perhaps, further characterisation of the population composition of the DFU would unveil the unique feature of every wound.

Chapter highlights:

DFU is predominantly found as polymicrobial consisted of mix growth including bacteria and fungi.

The predominant source of bacteria in DFU is mainly skin and gut.

Overlapping composition of broad group of bacteria was found regardless the severity of the wound.

3 Investigating the bacterial microbiomes of a cohort of diabetic foot ulcer patients

3.1 Introduction

Microbes including bacteria and fungi have a huge impact on the healing process of the wound. While open wounds can remain uninfected, over 50% of DFU will get infected (Armstrong et al., 2017; Edmonds et al., 2021; Prompers et al., 2007). In contrast to sterile anatomical sites, DFU is an open lesion that is not only exposed to colonisation by skin microbiota, but also microbes from other anatomical origins including oral cavity and gut, as well as the surrounding patient environment (Malone, Johani, et al., 2017). The exposure may potentially contribute to constant or intermittent introduction to a plethora of microbes into the wound.

Instead of living individually, microscopic imaging has revealed that bacteria are found to form biofilms in 60% of chronic wounds compared to only 6% in acute wounds (James et al., 2008). In another study by Johani K et al., all 65 DFUs are found to contain biofilms of either mono or multi-species (Johani et al., 2017). Additionally, more bacterial species are identified from chronic wounds in contrast to acute wound infection (James et al., 2008). In fact, bacteria in the wound is greatly more diverse when studied by 16S sequencing compared to the culture-based method (Jneid et al., 2017; Moon et al., 2021). This is contributed by the detection of more anaerobes and fastidious or slow-growing bacteria that are often difficult and challenging to grow under laboratory conditions (Jneid et al., 2017). Despite the polymicrobial nature of the wound, biofilm formation can appear as a single or multi-species community structure enclosed within the same wounds (Johani et al., 2017).

The prevalence is about 20% of the DFU will result in amputation (Armstrong et al., 2017; Dutra et al., 2019; McDermott et al., 2023). It has been shown that foot amputation is positively correlated with the severity of the wounds that were described according to the University of Texas classification system (Armstrong et al., 1998). However, studies on microbiome characteristics involving different grades and stages are limited to infected wounds. Therefore, it is crucial to characterise the microbiome of various grades and stages to compare the bacterial diversity in a spectrum of wound conditions.

Moreover, most studies did not account for the presence of fungi when delineating the wound microbiome therefore disregarding the importance of bacteria-fungi

interaction. Thus, the accuracy of 16S sequencing will allow a precise demonstration of species that are associated with fungi.

3.2 Hypothesis and aims

Bacterial populations interact and communicate with each other to thrive and cause diseases. Similarly, wounds are exposed to a vast species of bacteria that could hinder the healing process. A spatial characteristic of DFU wounds may predispose a group of more dominant species to prevail which may lead to a much more severe and chronic infection. This chapter will characterise the diversity of the 16S microbiome using Oxford Nanopore sequencing technology from DFU of different grades and stages classified based on the University of Texas wound classification system. Subsequently, the 16S microbiome will be explored with fungi isolated from culture.

Data from this chapter has been presented at the following conference and seminar:

Craig Williams, **Ahmed Bakri**, Jontana Allkja, Anna Cassey, Bryn Short, Jason Brown, Christopher Delaney, Paul Smith, Gordon Ramage. 'Microbiome analysis of non-healing diabetic foot ulcers reveal distinct patient specific microbiomes containing fungi that are minimally impacted by antibiotic therapy'. 34th ECCMID (European Congress of Clinical Microbiology and Infectious Diseases), Barcelona, Spain, April 2024.

Ahmed Bakri, Jontana Allkja, Anna Cassey, Bryn Short, Jason Brown, Christopher Delaney, Paul Smith, Craig Williams, and Gordon Ramage. 'Investigating the clinical importance of fungi in chronic wound'. School of Medicine, Dentistry and Nursing Post-graduate research day, May 2024.

3.3 Materials and methods

3.3.1 Sample demographics

A total of 349 wound swabs were processed for DNA extraction. Wound characteristics (grades and stages) are as summarised in Chapter 2.

3.3.2 DNA extraction

Dry wound swabs were thawed at room temperature before being processed. The swabs were extracted using MasterPure™ Yeast DNA purification kit (LGC Biosearch Technologies, UK) according to the manufacturer's protocol with some modifications. Evaluation of the extraction kit was performed by Dr. Bryn Short. Initially, the wound swabs were immersed in yeast lysis solution and sonicated for 15 minutes prior to the heating step at 65℃ for another 15 minutes. Once completed, the lysed cells were drawn out from the swabs by gently squeezed against microfuge tube to maximize the volume of the sample collected before discarding. Following that, samples were placed on ice for five minutes and were mixed with MPC protein solution by vortexing. Cellular debris was pelleted at >10000 rpm for 10 minutes and liquid supernatant was transferred to a new microfuge tube. Isopropanol was added with gentle mixing and then centrifuged at >10000 rpm for 10 minutes, leaving the DNA pellet at the bottom of the tube. The supernatant was removed and replaced with 70% ethanol to wash the pelleted DNA. The 70% ethanol was prepared in nuclease-free water (Life technologies, UK). Subsequently, ethanol was removed by pipetting, and then with a brief centrifugation to remove and dry the DNA pellet. Finally, TE buffer was added to suspend the DNA. All the reagents used for the extraction were supplied in the kit except for the isopropanol and ethanol.

3.3.3 DNA quantification and amplification by PCR

The extracted DNA were quantified using the NanoDrop ND-1000 spectrophotometer (Labtech International, Ringmer, UK) and standardised to 1 ng/µL with Nuclease-free water (Invitrogen). Briefly, the pedestals were cleaned with Whatman filter paper and primed with water to initialize. Next, 1 µL of TE buffer used to reconstitute the DNA at the end of the extraction protocol was used as a blank. Subsequent DNA samples were quantified at 260/230 nm ratio in ng/µL.

PCR reactions were performed by preparing the master mix consisting of the following ingredients as per reaction; 25 µL 2x VeriFi™ hot start mix (PCR Biosystems), 2 µL each primer and 16 µL of Nuclease free water.:

16S -27**F** - 5'- TTTCTGTTGGTGCTGATATTGCAGRGTTYGATYMTGGCTCAG – 3'
16S - 1492**R** 5'-ACTTGCCTGTCGCTCTATCTTCCGGYTACCTTGTTACGACTT-3'

Finally, a total of 5 µL of the standardised DNA (1 ng/µL) was added to the prepared master mix. The PCR reaction was performed with an initial denaturation of 30 seconds at 95℃. Subsequently, the reaction was run for 30 cycles with the following thermal profiles: denaturation for 15 seconds at 95℃; annealing for 15 seconds at 51℃; extension for 75 seconds at 65℃. Finally, the reaction was maintained at 65℃ for 10 minutes. The amplified DNA products were kept at 4℃ before the clean-up.

3.3.4 DNA clean up

The amplified DNA products were transferred into a clean DNA Lo-Bind Eppendorf tube. In a DNA Lo-bind Eppendorf tube, 25 µL of AMPure XP beads were added with mix pipetting followed by five minutes of incubation at room temperature on a Hulla mixer. The samples were then centrifuged briefly before pelleting on a magnet. The supernatant was removed while keeping the tube on the magnet and the pellet was washed with 200 µL of freshly prepared 70% ethanol twice by discarding the ethanol before the second wash. Subsequently, the tubes were centrifuged briefly then any residual ethanol was removed by pipetting and the pellets were left to dry for ~30 seconds. After that, the pellets were then resuspended in 10 mM Tris-HCl pH 8.0 with 50 mM NaCl (Qiagen, UK) and incubated for two minutes before pelleting on a magnet. Finally, the eluate containing the DNA was removed and retained in clean DNA Lo-Bind tubes. The end products were measured using a Qubit™ fluorometer and standardised to 5 ng/ µL for barcoding.

3.3.5 DNA quantification using Qubit™

All the cleaned-up DNA products were quantified using Qubit™ dsDNA Broad Range (BR) Quantification assay kit (ThermoScientific, UK). A master mix containing the fluorescence dye, and the buffer provided in the kit were prepared at 2 µL and

198 µL for each sample respectively. Subsequently, 199 µL of the prepared master mix were aliquoted into the Qubit tube followed by the addition of 1 µL DNA. The tubes were incubated in the dark for two minutes before reading. Before the measurement, the Qubit™ 4 fluorometer was calibrated using the low (Standard 1) and high (Standard 2) standards provided. All the tubes were read and recorded in ng/µL.

3.3.6 DNA barcoding

The DNA barcoding was carried out using PCR barcoding expansion 1-96 (Oxford Nanopore Technologies, UK). A master mix was prepared for all samples with the following ingredients and volumes; Hot start Taq (25 µL), barcode (1 µL), Nuclease-free water (19 µL) and previously standardised DNA of 5 ng/µL (5 µL). Next, the barcoding reactions were run in a thermocycler with the following thermal profiles; Initial denaturation for 30 seconds at 95℃ followed with 15 cycles of denaturation and annealing for 30 seconds at 95℃ and 62℃ respectively, extension for 75 seconds at 72℃ and final extension at 65℃ for 10 minutes. Following the reaction, the barcoded samples were maintained at 4℃ before the clean-up. The DNA clean-up was performed as described above. All the barcoded samples were then pooled into a Lo-Bind Eppendorf tube with a max of 1000 ng DNA from a total pool. The pooled DNA was cleaned up and eluted with elution buffer to get 49 µL of a final pooled DNA.

3.3.7 DNA End-prep

The end-prep was carried out using Ligation Sequencing Amplicons V14 (SQK-LSK114, Oxford Nanopore Technologies, UK) according to the manufacturer protocols. Initially, the DNA Control sample (DCS) was thawed at room temperature before a brief spun followed by mix pipetting. The NEBNext Ultra II End-Prep reaction buffer was mixed properly to dissolve all precipitate before use. The end-prep reaction was prepared in a 0.2 mL PCR tube by adding 1 µL of DNA CS into 49 µL of cleaned pooled DNA. The Ultra II end-prep reaction buffer and Ultra II end-prep enzyme mix were then added in 7 µL and 3 µL, respectively, making a total reaction volume of 60 µL. The reaction was gently mixed by pipetting and spun down followed by incubation in a thermocycler at 20℃ and 65℃ for five minutes each. Once finished with the incubation, the DNA was

transferred into a clean Lo-bind Eppendorf tube with the addition of 60 µL of AMPure XP Beads and incubated on a Hulla-mixer for five minutes. The DNA was then pelleted on a magnet and washed twice with 80 % ethanol. Finally, the pellet was resuspended in 61 µL of Nuclease-free water and the eluate containing the DNA was transferred into a clean Lo-bind Eppendorf tube by placing on a magnet.

3.3.8 Adaptor ligation and clean-up

All the reagents for this step were added sequentially with the mix pipetting in between each addition. Firstly, 25 µL of the ligation buffer (LNB) was added to the DNA obtained from the previous step in a DNA Lo-bind Eppendorf tube. Following that, the NEBNext Quick T4 DNA Ligase and Ligation adapter (LA) were added in order at 10 µL and 5 µL volume, respectively. A total of 100 µL reaction volume was gently mixed followed by a brief spun and incubated for 10 minutes at room temperature. The reaction was then added with 40 µL of AMPure XP Beads (AXP) and incubated on a rotator mixer for five minutes. Subsequently, the DNA was pelleted on the magnet and the supernatant was removed and discarded. After that, the pellet was washed twice with 250 µL Short Fragment Buffer (SFB) and eluted with 15 µL of Elution buffer (EB). Finally, the DNA library was transferred to a clean DNA Lo-bind tube and quantified with Qubit™ fluorometer. The final library concentration was adjusted to be in a range of 35 – 50 fmol in 12 µL of elution buffer. The DNA library was stored at 4℃ until ready for loading into a flow cell.

3.3.9 Priming and loading the flow cell

Before loading the flow cell (Oxford Nanopore Technologies, UK), the priming mix was prepared according to the manufacturer's protocol. Briefly, the priming mix was prepared by adding the Flow Cell Flush (FCF), Bovine Serum Albumin (BSA) at 50 mg/mL and Flow Cell Thether (FCT) at 1170 µL, 5 µL and 30 µL respectively. An 800 µL priming mix was then loaded into the flow cell and waited for five minutes before loading another 200 µL of the priming mix. While waiting for the second priming, the previously prepared DNA library was prepared by adding with Sequencing Buffer (SB) and the Library Beads (LIB) at 37.5 µL and 25.5 µL, respectively. Finally, the library was loaded into the flow cell immediately after the second priming step. The sequencing was then set up on the sequencing device

using the Ligation sequencing kit SQK-LSK114 (Oxford Nanopore Technologies, UK). Optimisation of the Nanopore sequencing was performed by Dr. Bryn Short. An overview of the major work processed in this chapter is presented in Figure 3.1.

3.3.10 Data acquisition

Raw input data (raw electrical signal) from the sequencing device were converted to FASTQ files (nucleotide sequence) by using a MinKnow basecalling software R10.4.1. Basecalling setting was processed at a super accuracy configuration on MinKnow software. Following this, the taxonomic level of the sequencing reads was analysed using a closed reference operational taxonomic unit (OTU) clustered based on the Emu software. The low counts were removed and filtered out from the final OTU count table. This process was supported by Dr Christopher Delaney.

3.3.11 Microbiome data analysis

Analysis of the microbiome data was done on MicrobiomeAnalyst 2.0 as described previously (Chong et al., 2020). Initially, the data were uploaded to the MicrobiomeAnalyst 2.0 platform according to the specific data format. The OTU counts table was uploaded in tab-delimited text file (.txt) format while the metadata and taxonomy table were both in comma-separated values (.csv) format. Data normalization was performed based on the rarefying to the minimum size of the library after filtering the samples with low reads. Community profiling and visual exploration were initially carried out on MicrobiomeAnalyst and data was transferred to GraphPad Prism for high-resolution figures.

3.3.12 Statistical analysis

The microbiome data were analysed using MicrobiomeAnalyst 2.0 platform (Chong et al., 2020) and visualization of the relative abundance was made using GraphPad Prism (Version 10.2.2, La Jolla , CA, USA). Sequencing data were rarefied based on the lowest read count of the samples included in a particular analysis. Alpha-diversity of different grades and stages were analysed using Chao1 and Shannon indices that were based on Mann – Whitney/ Kruskal – Wallis pairwise comparison. Furthermore, ß- diversity was analysed using Bray – Curtis index with pairwise PERMANOVA. Bacterial loads of different grades and stages were analysed using

Kruskal – Wallis test with Dunn's multiple comparison test. A p values of < 0.05 was considered as statistically significant for all the analysis.

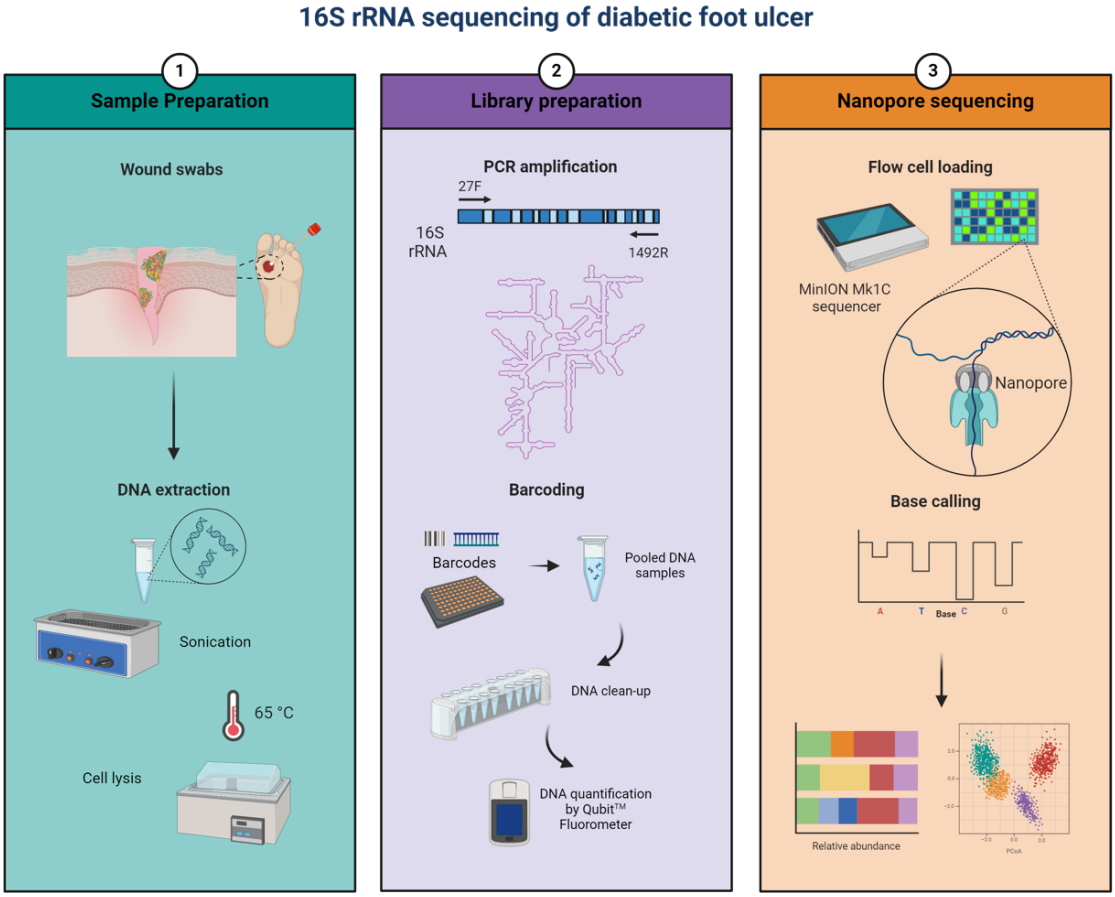


Figure 3.1 Overview of the work process on 16S microbiome by Nanopore sequencing. Created with BioRender.com.

3.4 Results

3.4.1 Clinical sample procurement

A total of 349 wound swabs from 127 patients were processed in this study between August 2021 and January 2023 (Figure 3.2). The patients who were involved in this study had contributed to a range of 1-10 wound swabs.

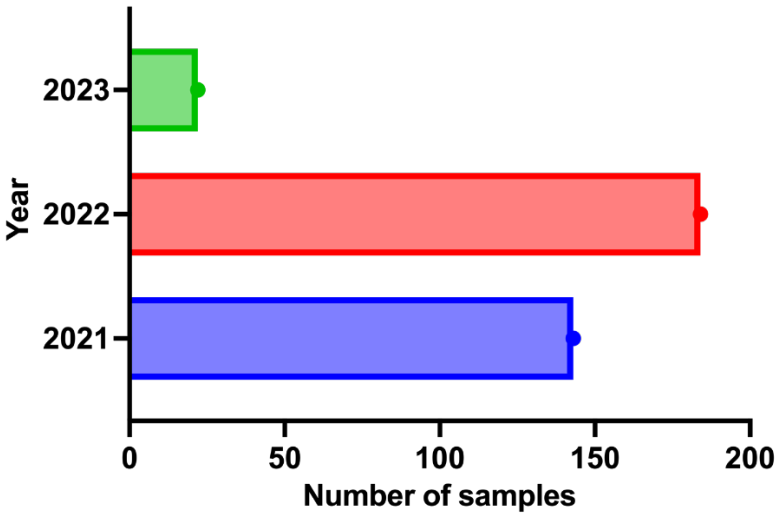


Figure 3.2 Cumulative of wound swabs collected in this study.

3.4.2 Bacterial load in the wounds

qPCR was used to to quantify the 16S burden in the wounds. There was no statistical difference observed between grades ($p = 0.2385$) and stages ($p = 0.1981$) of the wounds with respect to estimated bacterial burden (Figure 3.3).

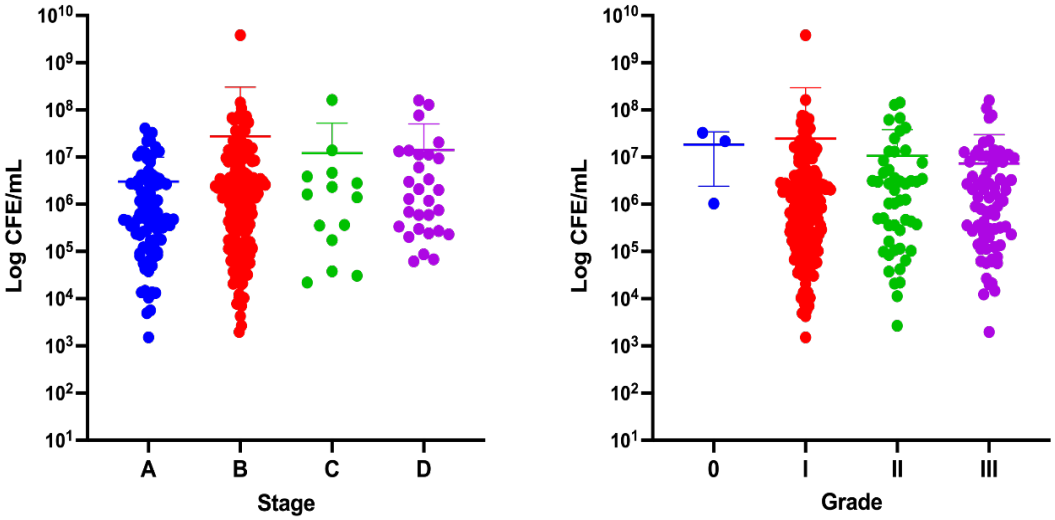


Figure 3.3 Comparable bacterial load across all grades and stages. Bacterial load as measured based on 16S qPCR does not show any statistical difference across all stages and grades of wounds.

3.4.3 Heterogenous baseline microbiome

A total count of 7,760,252 reads with an average of 23,234 reads per sample was obtained from 334 DFU swabs. Fifteen samples with zero reads were excluded from the analysis. Three samples of low counts in comparison to most of the samples were filtered out from further analysis. Upon data filtering, 907 OTU features were identified. For the analysis of the baseline microbiome, sequencing depth was rarefied to 1199 reads. Samples of grade 0 were removed from the analysis when comparing the ß-diversity due to incomparable sample size to other grades for the analysis to perform correctly.

Of the microbiomes from the first visits, *Staphylococcus* and *Corynebacterium* were the most predominant genera, at 17.09% and 12.23% of relative abundance (RA), respectively (Figure 3.4). These two taxa were the only taxa that were above 10% RA among others. Other genera in descending order include *Anaerococcus* (6.22%), *Streptococcus* (5.38%), *Prevotella* (4.27%) and *Finegoldia* (3.37%). Taxa distribution for each patient's first visit is shown in Figure 3.5. Since some patients have multiple swabs that contain the same taxa across the visits, therefore comparing the baseline microbiome between patients was performed on the swabs taken from the first visit to avoid biases of the recurring taxa.

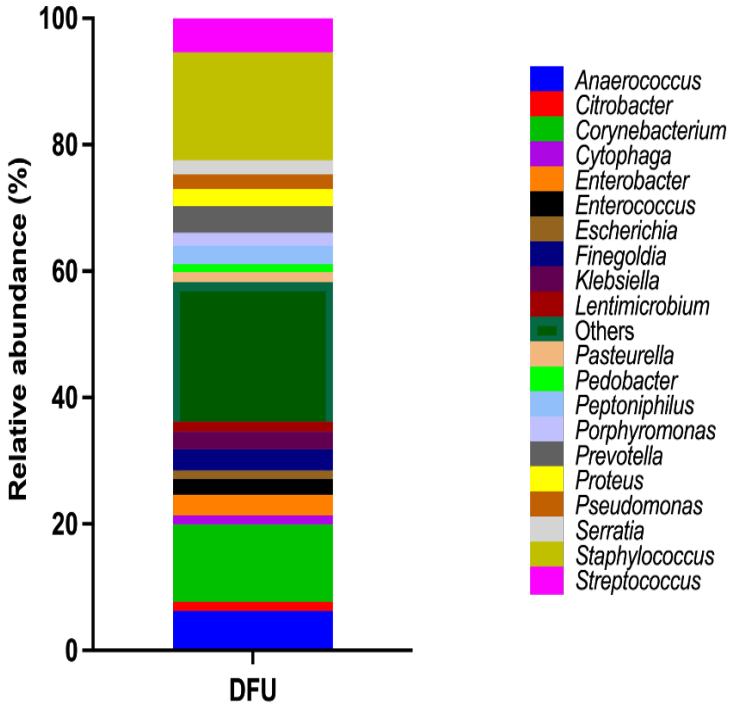


Figure 3.4 Most common taxa of baseline wound microbiome. The stacked bar plot shows the composition based on the relative abundance of the most common genera identified in the wounds collected during the first visit.

Next, to investigate the within-sample diversity (α-diversity), Chao1 and Shannon diversity indices were used that measure the richness, richness and evenness respectively. Richness represents the number of taxa that are present, while evenness measures the abundance of the taxa. The swabs of the first visit account for different grades and stages as previously described in Chapter 2. There were no significant differences in the α-diversity between different grades and stages of the first visit swabs (Figure 3.6). Similarly, based on the Bray-Curtis dissimilarity index to measure the ß-diversity, no statistically significant difference was observed for swabs of different grades and stages (Figure 3.7).

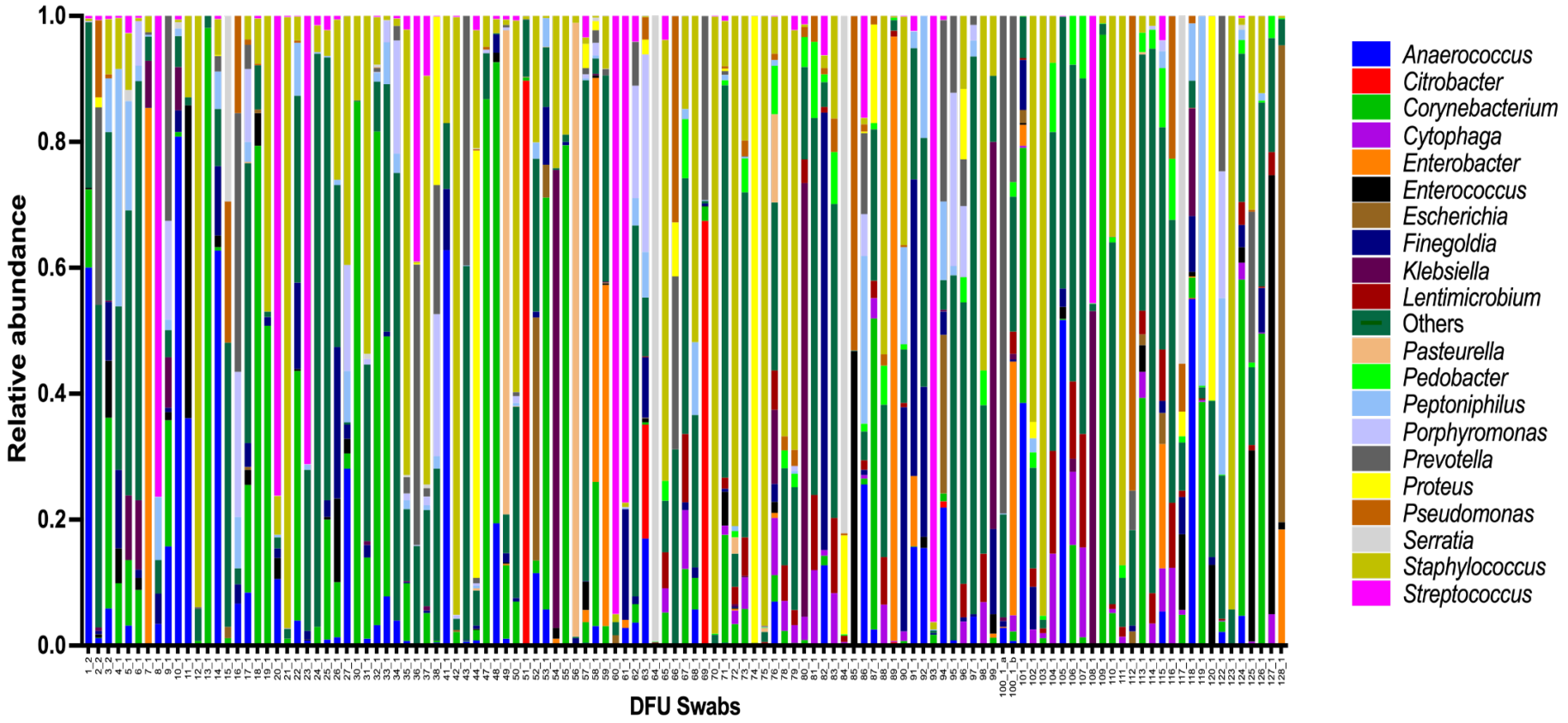


Figure 3.5 Patients first visit microbiome. The stacked bar plot shows the relative abundance of the individual composition of the taxa identified at a genus level from wounds collected during the first visit.

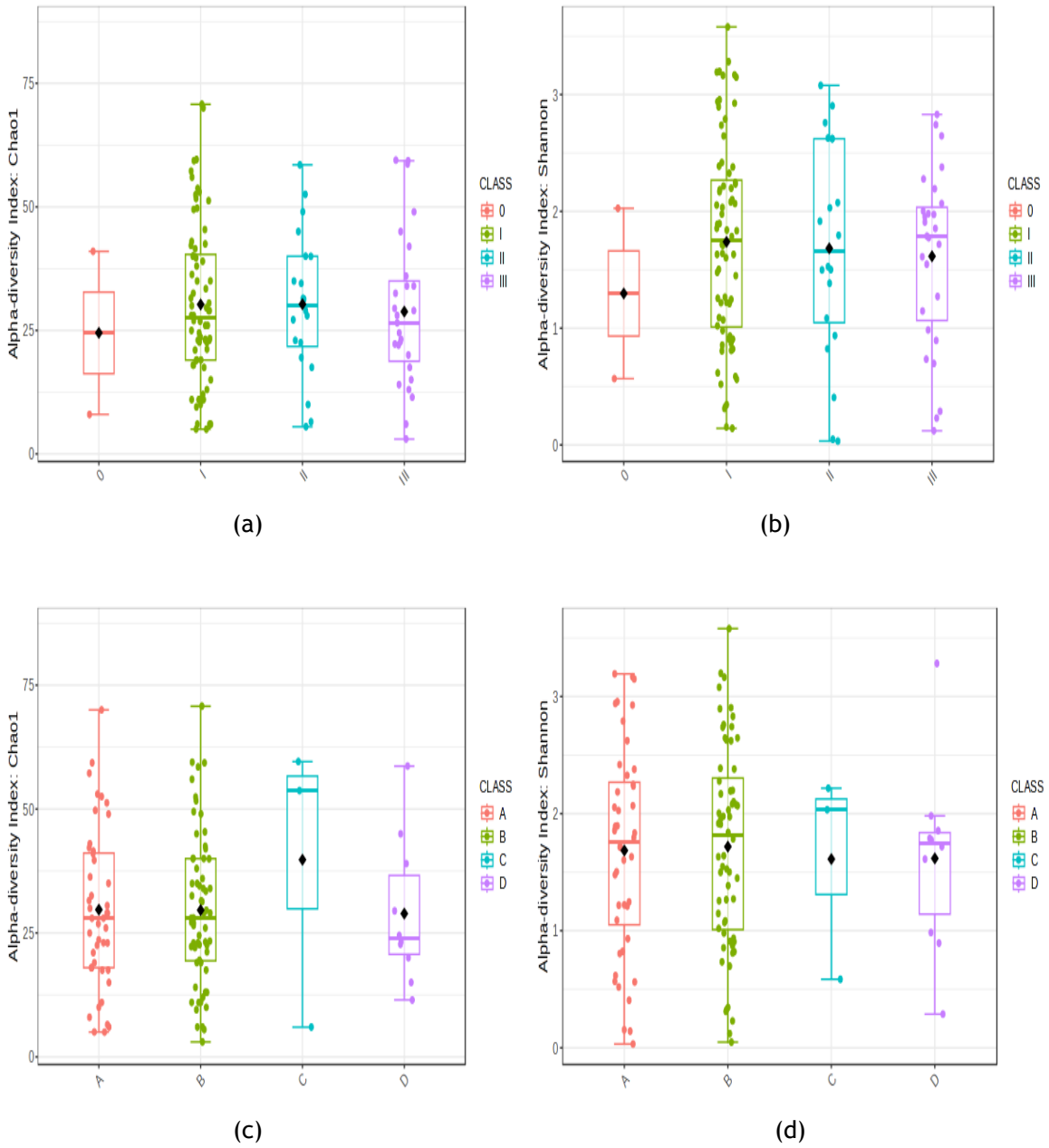


Figure 3.6 Alpha - diversity of baseline microbiomes. Box plots show the bacterial diversity within the sample as measured using Chao1 and Shannon indices across different grades (a, b) and stages (c, d). No significant difference was observed for both indices based on Kruskall-Wallis with post-hoc pairwise comparisons.

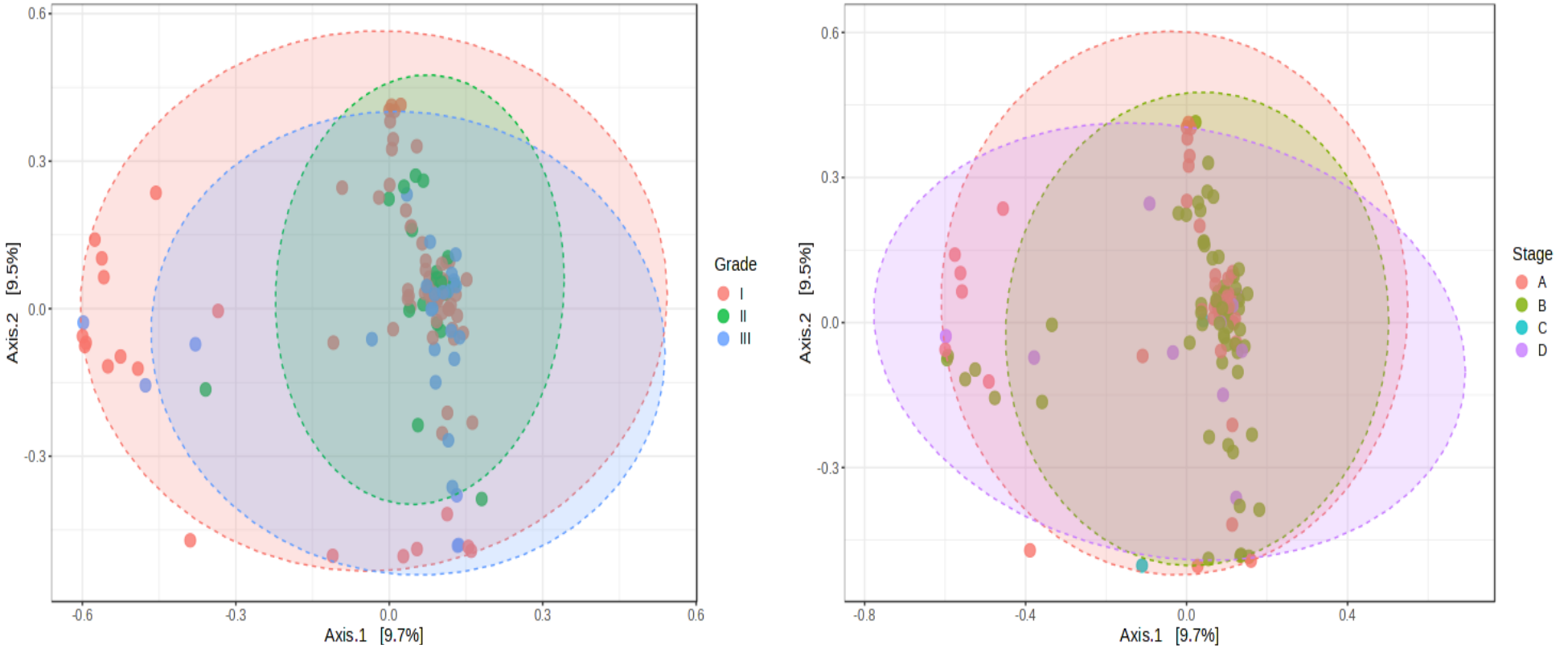
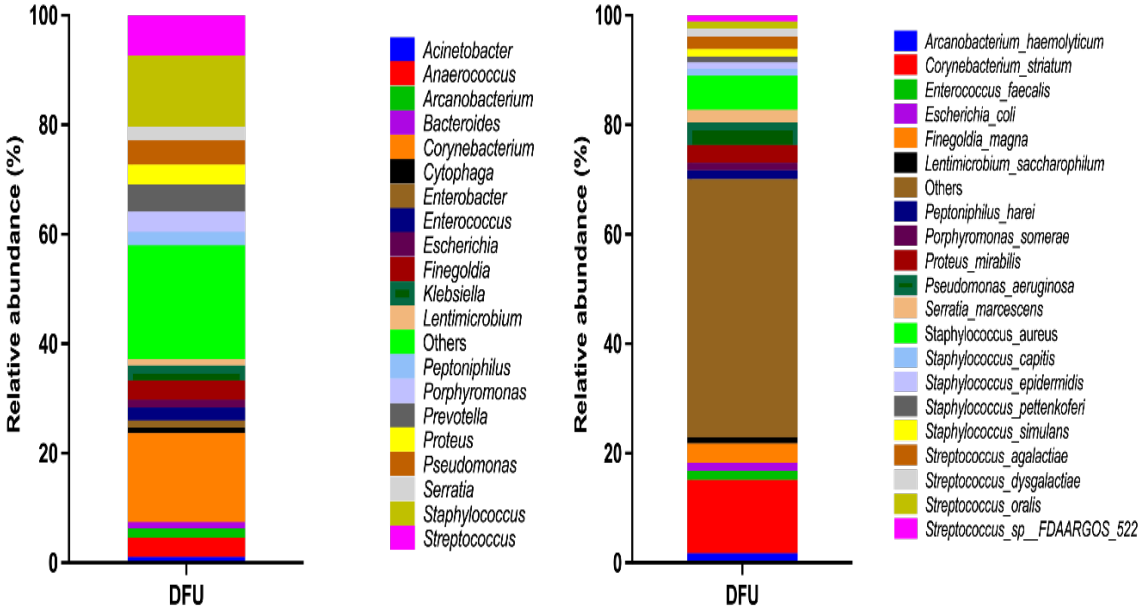


Figure 3.7 Beta - diversity of baseline microbiome. PCoA plot with Bray- Curtis dissimilarity does not show any clustering between the wounds of different grades (a) and stages (b) from the samples of the first visit.

3.4.4 All samples microbiome

As the baseline microbiome only represents the snapshot of the microbiome from every patient at a single time point, the subsequent analysis included a larger cohort with all the swabs collected in this study were considered. Analysis was based on the filtered data as described above in 3.4.3. For the analysis of all samples' microbiomes, sequencing depth was rarefied to 1027 reads. In addition, due to only three samples in grade 0, these samples (30_1, 62_1 and 10_11_a) were removed from the analysis when comparing the ß-diversity due to incomparable sample size to other grades for the analysis to be performed correctly.

All samples microbiome appeared to be unaffected by the severity of the wounds. The top major genera and species identified in all swabs were presented in Figure 3.8. Genus of *Corynebacterium*, *Staphylococcus* and *Streptococcus* were the most prevalent with RA of 16.23%, 13.04% and 7.36%, respectively. Other taxa in descending order of RA included *Prevotella*, *Pseudomonas* and *Porphorymonas* with 4.97%, 4.43% and 3.71%, respectively. Upon speciation of the taxa, *Corynebacterium striatum* was the most predominant within all the samples followed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, with 13.44%, 6.18% and 4.11% RA respectively. *Finegoldia magna* was the most abundance species among the obligate anaerobes (3.53%). Speciation also shows the top taxa were predominated by the *Staphylococcus* and *Streptococcus* species.



(a) (b)

Figure 3.8 Most common taxa in DFU microbiome. Stacked bar plots show the relative abundance of the bacterial composition according to genus (a) and species (b) of all the DFU.

All samples' microbiomes appeared to be unaffected by the severity of the wounds upon analysing the α and ß-diversity indices. All grades and stages microbiome were found to be similar to each other (Figure 3.9). This is also evidenced from the PCoA plot of the ß-diversity that shows no clustering based on grades and stages of the wounds (Figure 3.10).

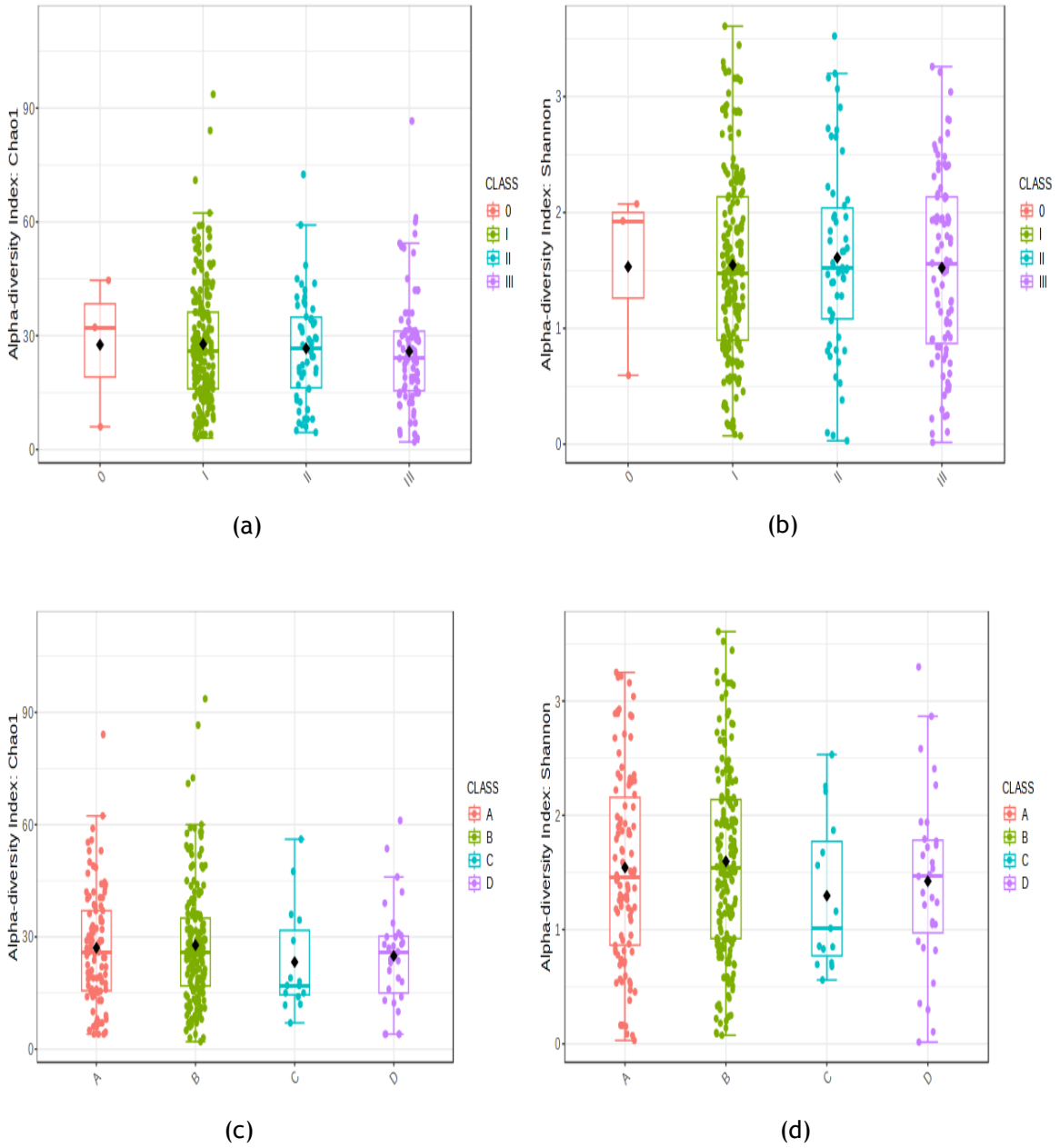


Figure 3.9 Alpha - diversity of all the wounds. Box – plots show the bacterial diversity within the samples as measured using Chao1 and Shannon indices across different grades (a, b) and stages (c, d)

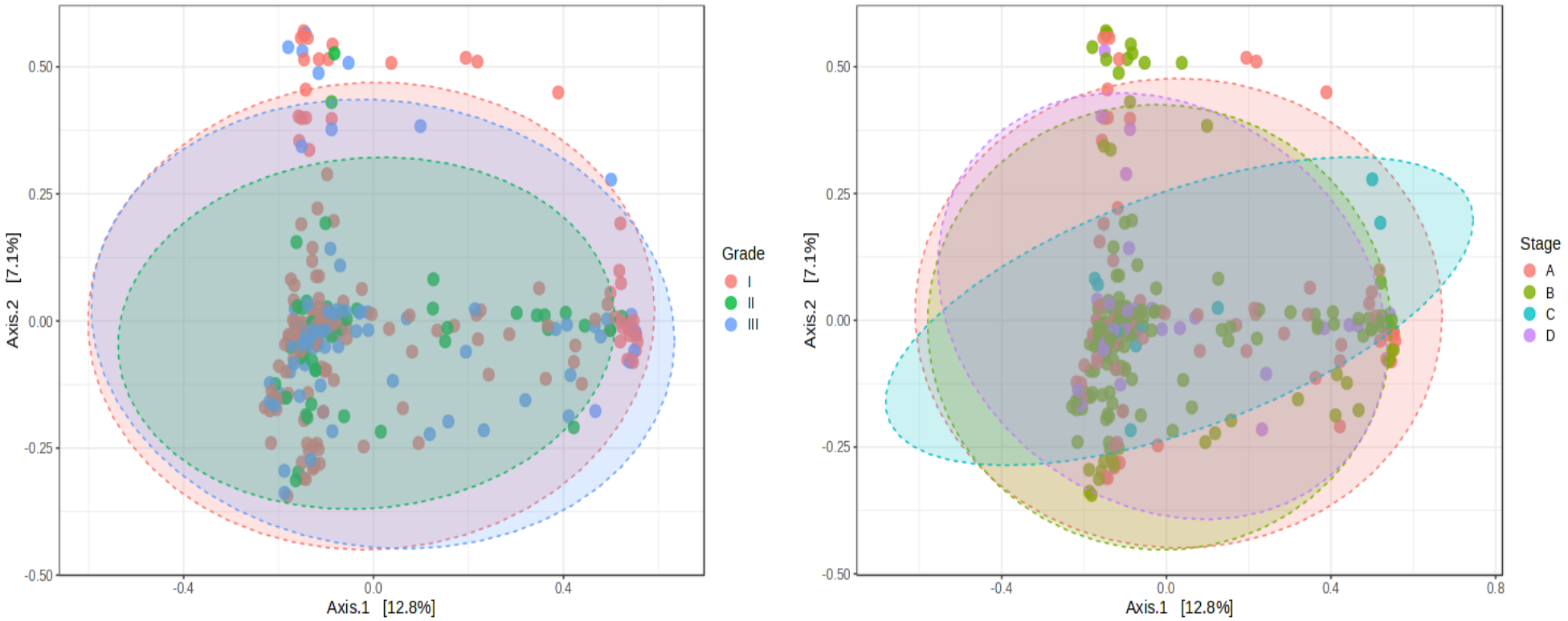


Figure 3.10 Beta - diversity of all the wounds. PCoA plots with Bray – Curtis dissimilarity do not show any clustering between wounds of different grades (a) and stages (b).

3.4.5 Superficial infected versus non-infected wounds

Despite no distinct clusters being observed between grades and stages as described above, the next analysis was to compare the microbiome according to grades and stages that show comparable in sample size. Firstly, Grade I (superficial) was compared between non-infection (Stage A) versus infection (Stage B) wounds. For this analysis, the sequence reads were rarefied to 1199 reads as this was the lowest reads in this cohort.

The microbial composition for non-infected and infected wounds was presented in Figure 3.11 (genus level) and Figure 3.12 (species level). Top genera from non-infected wounds were predominated by *Corynebacterium*, *Staphylococcus* and *Streptococcus* with RA of 19.49%, 19.36% and 4.99% respectively. Other taxa included *Anaerococcus* (3.2%), *Pasteurella* (3.08%), *Enterobacter* (2.64%) and *Finegoldia* (2.64%). On the other hand, *Streptococcus*, *Corynebacterium* and *Staphylococcus* were the predominant taxa of the infected wounds in descending order of RA at 14.12%, 14.08% and 13.69% respectively. Standing out in the infected wound was the presence of *Bacteroides* at 1.08% compared to only 0.01% in the non-infected wounds (taxa not included in the Figure). Upon analysing the data at a species level, *C. striatum* was the predominant species in both conditions followed by *S. aureus* (9.49%) and *P. multocida* (2.96%) in non-infected stage and *P. aeruginosa* (6%) and *S. aureus* (5.68%) in infected stage.

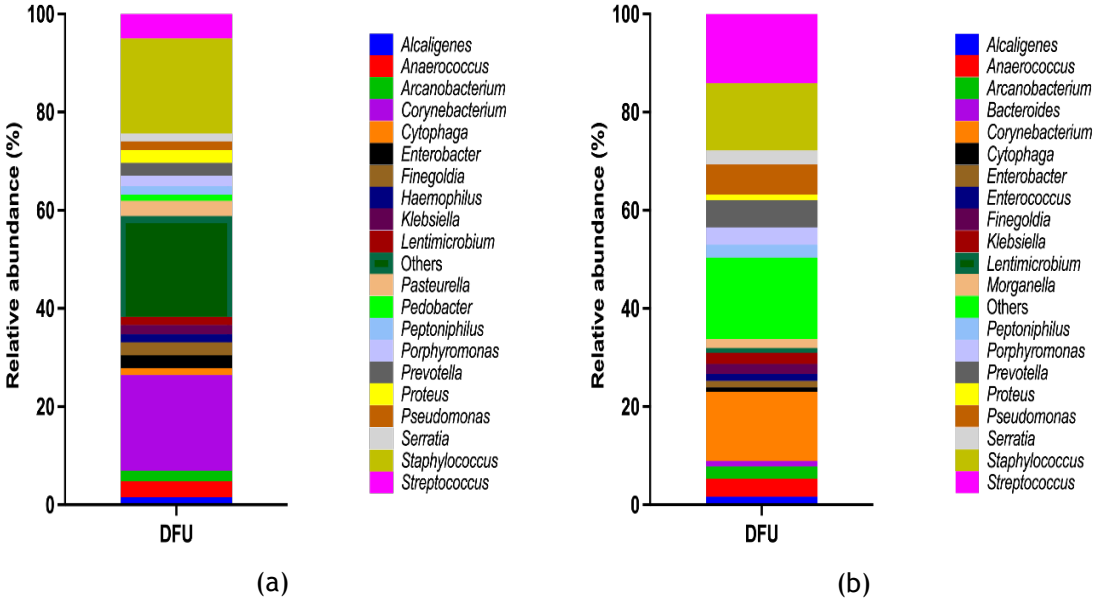


Figure 3.11 Taxa (genus) associated with non-infected and infected superficial wounds. Stacked bar plots show the relative abundance of bacterial composition from the non-infected (a) and infected (b) superficial wounds. The non-infected and infected superficial wounds denote the AI and BI of grades and stages based on the University of Texas classification system respectively.

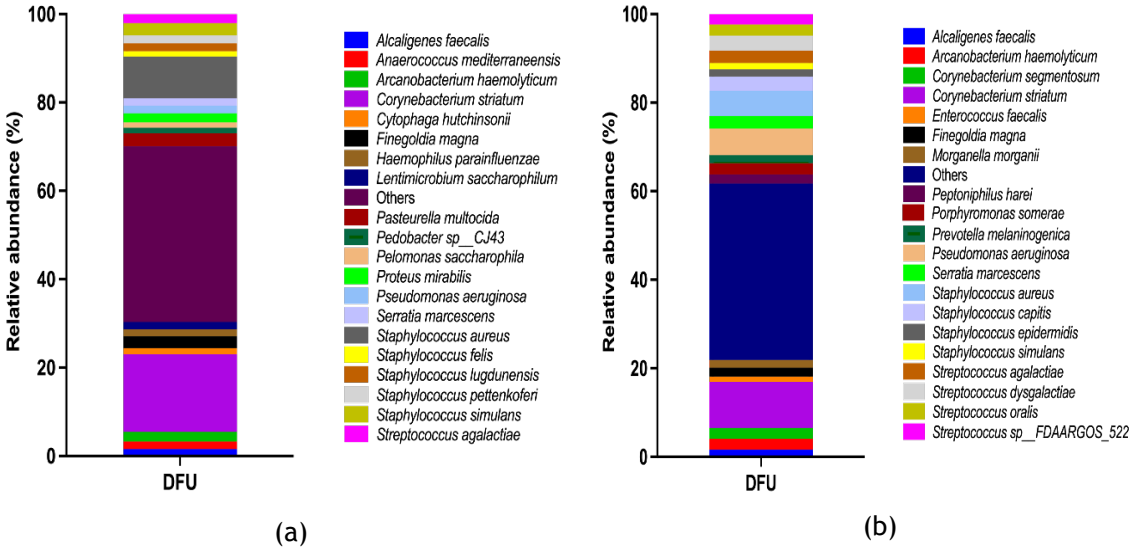
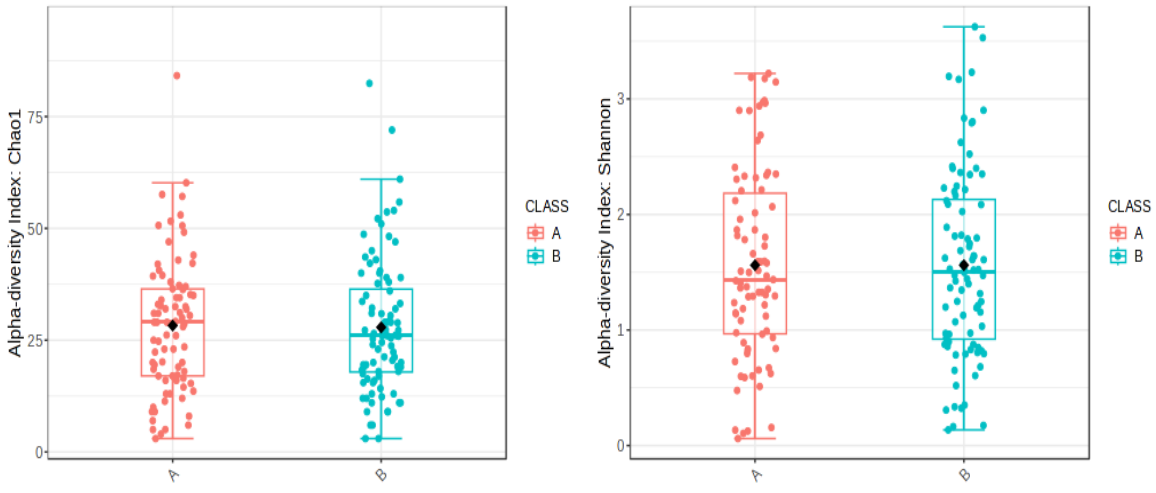


Figure 3.12 Taxa (species) associated with non-infected and infected superficial wounds. Stacked bar plots show the relative abundance of bacterial composition from the non-infected (a) and infected (b) superficial wounds. The non-infected and infected superficial wounds denote the AI and BI of grades and stages based on the University of Texas classification system respectively.

In order to assess for diversity in these stages, α and ß-diversity were analysed. No significant difference was observed for within sample diversity (Figure 3.13).

However, a statistically significant difference (p =0.037) was observed for ß-diversity between these two conditions (Figure 3.14).



(a) (b)

Figure 3.13 Alpha - diversity of non-infected and infected wounds. Box – plots show the bacterial diversity within the samples as measured using Chao1 (a) and Shannon (b) indices for wounds of AI and BI respectively.

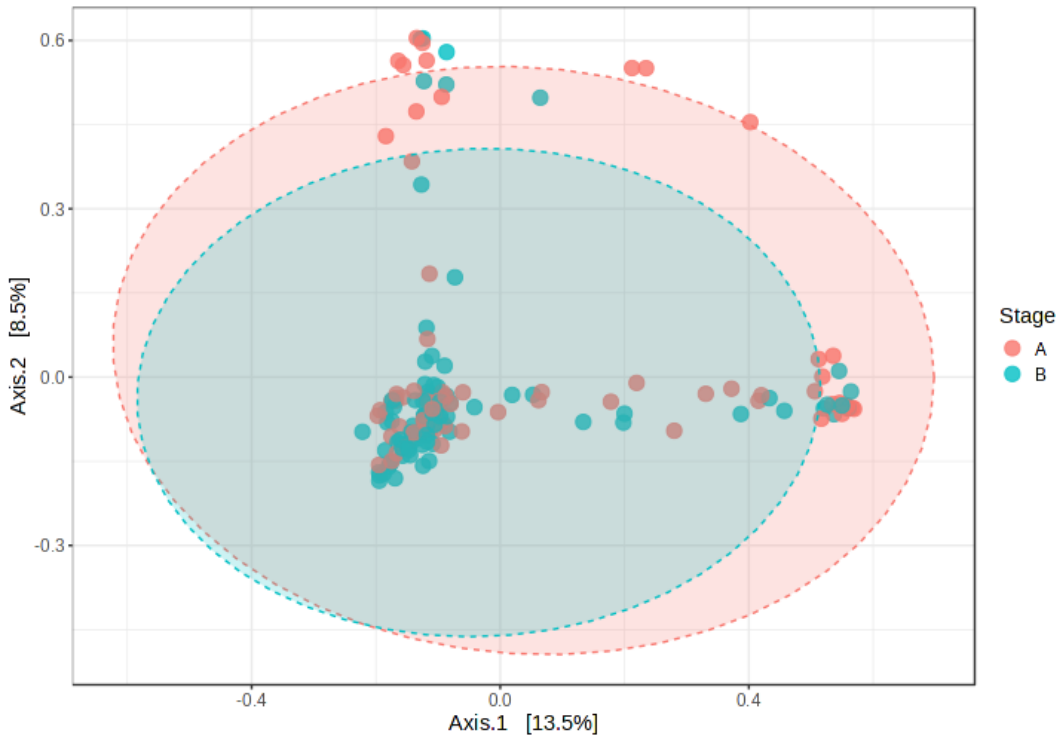


Figure 3.14 Beta- diversity of non-infected and infected wounds. PCoA plots with Bray – Curtis dissimilarity show a statistically significant difference (p = 0.037) between wounds from AI and BI.

To further analyse the taxa that contribute to the differences, LefSe analysis was performed. Non-infected wounds showed an increased in Coagulase negative *Staphylococcus* (*S. lugdunensis* and *S. pettenkoferi*) while infected superficial wounds were enriched with obligate anaerobes and facultative anaerobes (Figure 3.15).

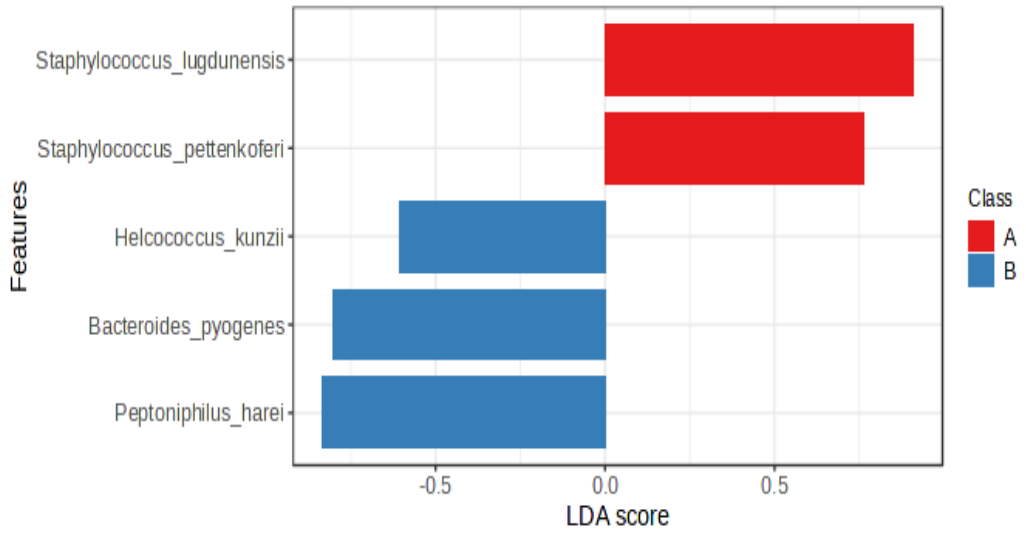


Figure 3.15 Taxa enriched in non-infected and infected wounds. LEfSe analysis identified taxa that were more abundant in stage A (red) and stage B (blue).

3.4.6 Infected superficial (Grade I) and deep wounds (Grade II and III)

DFU infection can spread into deeper tissue that involves tendons and bone which indicate a more severe infection. The next analysis is to compare the microbiome of infected wounds based on the depth (Figure 3.16). The sequencing reads were rarefied to 1027 reads before proceeding with the analysis.

Firstly, superficial wounds were found to be predominated with *Corynebacterium* (14.15%), *Streptococcus* (14.13%) and *Staphylococcus* (13.73%). Grade II involves wounds that penetrate the tendon. The most abundant taxa at the genus level identified in grade II were *Corynebacterium* (11.17%) followed by *Staphylococcus* (8.36%) and *Prevotella* (7.7%). Similarly, grade III was found to be predominated by *Corynebacterium* (20.07%), *Staphylococcus* (10.68%) and *Prevotella* (6.68%).

At the species level, *Corynebacterium striatum* was the highest abundance at all wound depths with RA of 10.13%, 9.7% and 16.87% for grades I, II and III,

respectively. In grade I wounds, *P. aeruginosa* (6.01%) and *S. aureus* (5.67%) were the next most abundant species identified. This was similarly observed in grade III wounds but with a slightly higher abundance of *S. aureus* (5.9%) compared to *P. aeruginosa* (5.13%). On the other hand, grade II shows *S. agalactiae* (4.88%) and *S. marcescens* (4.8%) as the most common following *C. striatum*.

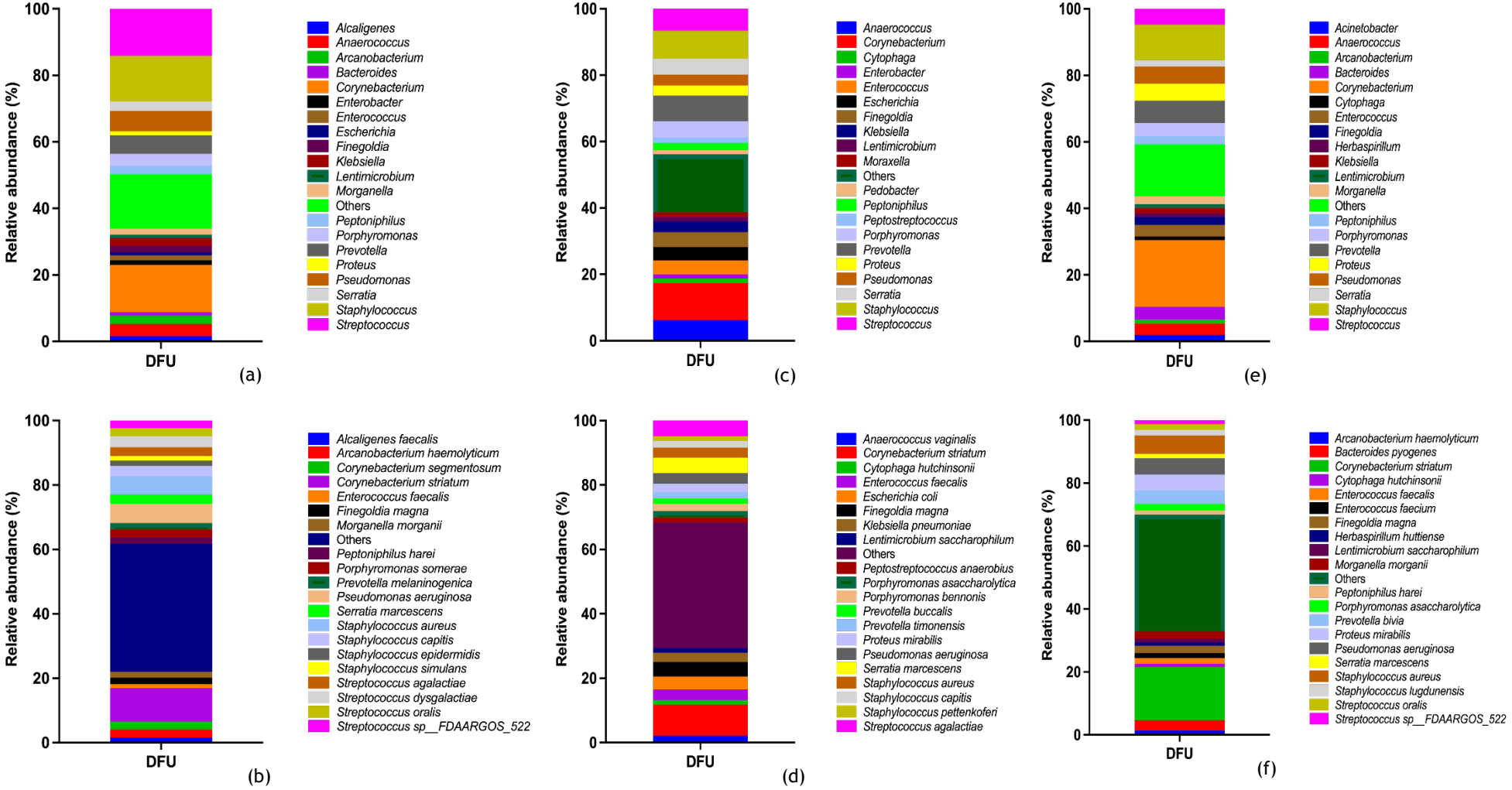
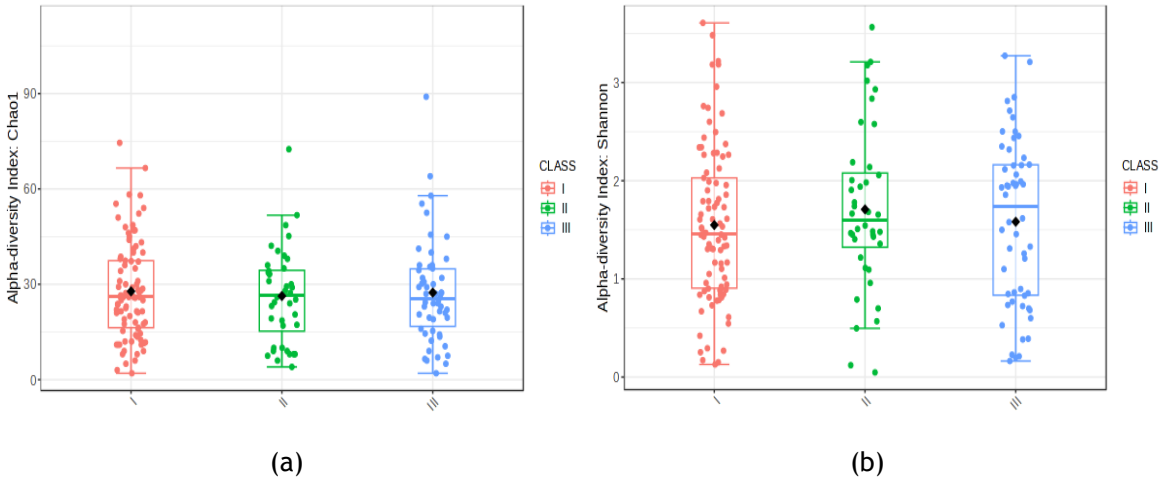


Figure 3.16 Taxa (genus and species) associated with wound depth. Stacked bar plots show the relative abundance of bacterial composition from infected superficial (a, b), tendon (c, d) and bone (e, f).

To further analyse the diversity based on the wound depth, α and ß-diversity were performed. However, both diversity indices did not show any statistically significances differences (Figure 3.17 and Figure 3.18).



(a) (b)

Figure 3.17 Alpha- diversity of infected wounds from superficial, tendon and bone. Box – plots show the bacterial diversity within the samples as measured using Chao1 (a) and Shannon (b) indices from infected wounds of grades I, II and III.

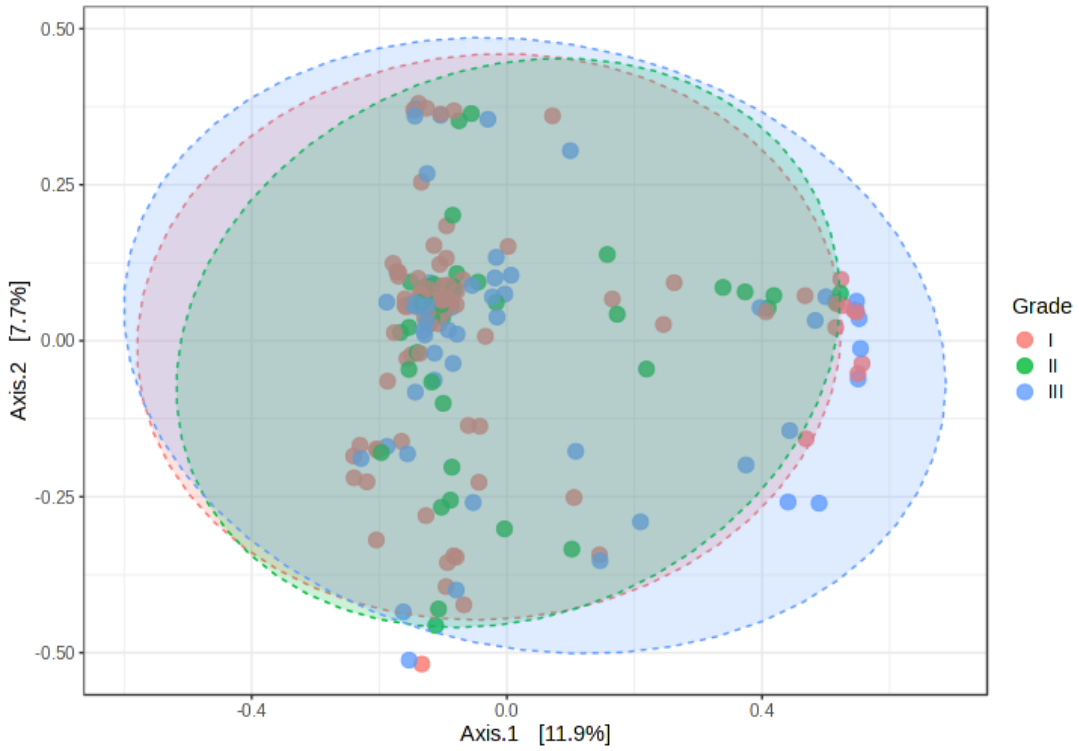


Figure 3.18 Beta-diversity of infected wounds from superficial, tendon and bone. PCoA plots with Bray – Curtis dissimilarity do not show a significant difference between the infected wounds of grades I, II and III.

3.4.7 Ischemic versus Infected-ischemic wounds

An ischemic wound was associated with insufficient blood flow resulting in low nutrients and oxygen delivered to the tissue. Anaerobes were mostly associated with this low-oxygen environment. Therefore, the next analysis will compare the microbiomes associated with ischemic wounds. For this analysis, the sequencing reads were rarefied to the lowest read at 1836 reads.

At genus level composition, ischemic wounds (stage C) were predominated by *Corynebacterium* (17.43%), *Proteus* (10.41%) and *Finegoldia* (9.56%) while in stage D, wounds were predominated by *Staphylococcus* (11.57%), *Corynebacterium* (10.92%) and *Finegoldia* (8.13%) (Figure 3.19). Both stages shown the prevalence of obligate anaerobes (*F. magna*). At the species level, *C. striatum* (16.69%), *P. mirabilis* (10.41%) and *F. magna* (9.56%) were found to be the most prevalent in stage C while *C. striatum* (10.18%), *F. magna* (8.13%) and *S. aureus* (8.12%) were predominated in stage D (Figure 3.20).

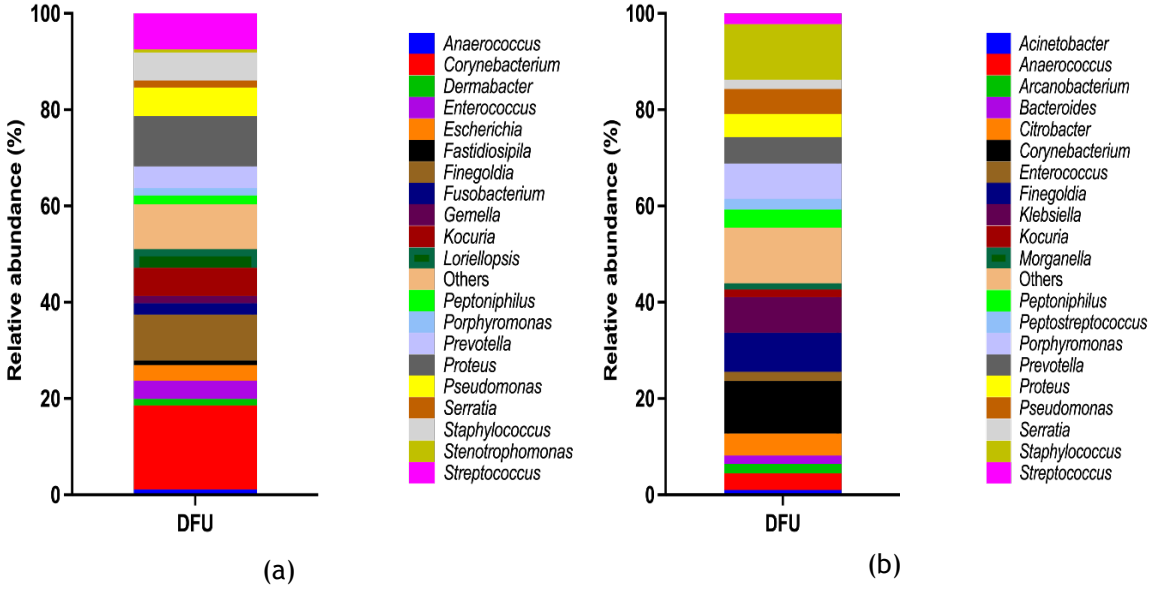


Figure 3.19 Taxa (genus) associated with ischemic and infected-ischemic wounds. Stacked bar plots show the relative abundance of bacterial composition from the ischemic (a) and infected-ischemic wounds (b).

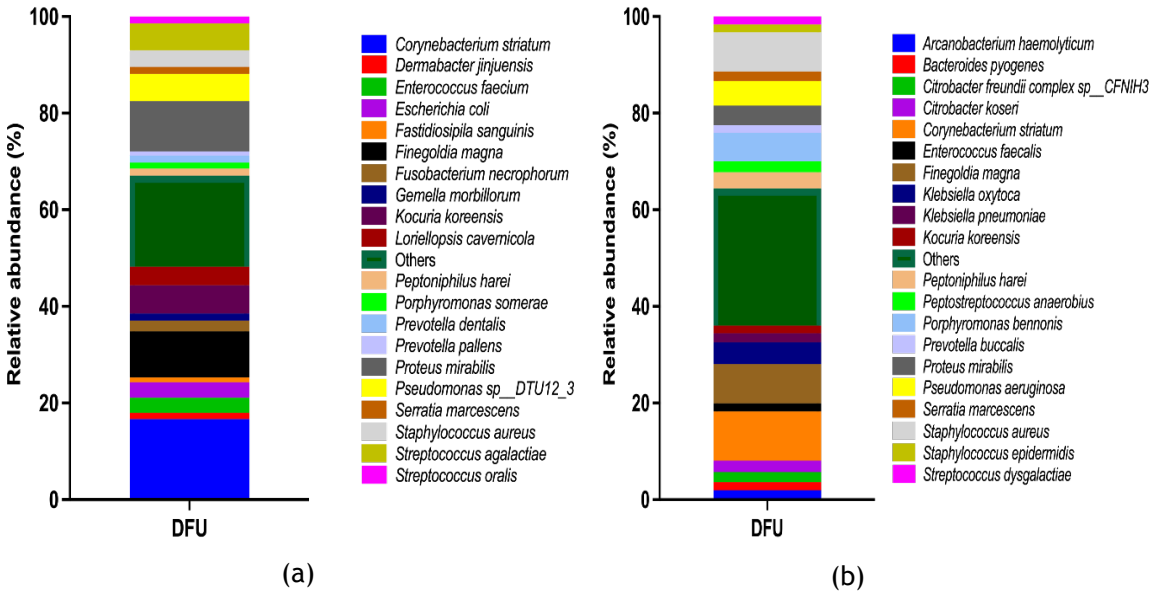


Figure 3.20 Taxa (species) of ischemic and infected-ischemic wounds. Stacked bar plots show the relative abundance of bacterial composition from the ischemic (a) and infected-ischemic wounds (b).

Both α and ß-diversity were performed for these wound conditions. Both α and ß-diversity did not show any significant difference as shown in Figure 3.21 and Figure 3.22, respectively. There was no distinct clustering associated between these wound conditions.

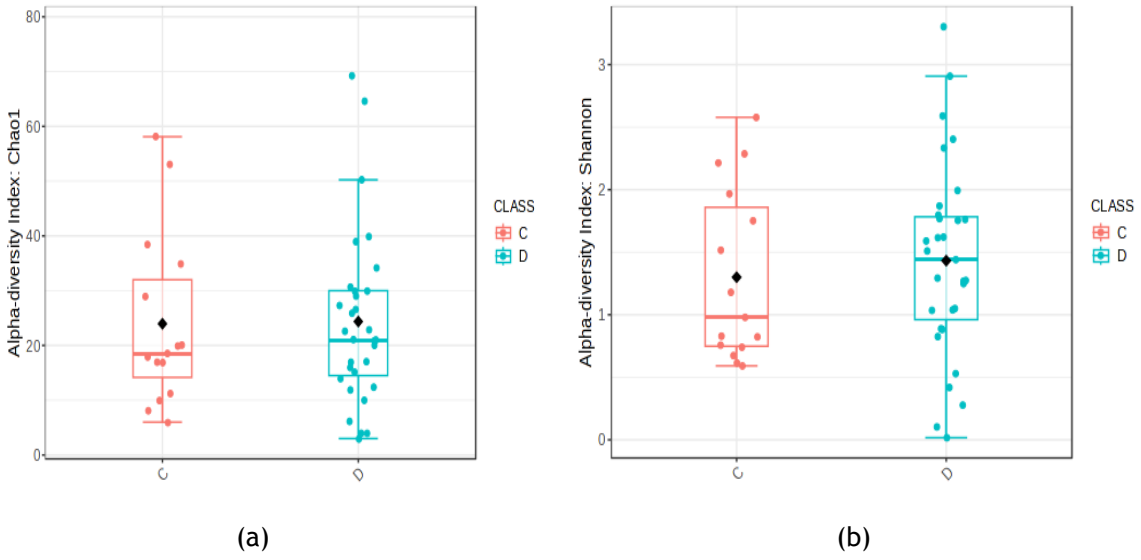


Figure 3.21 Alpha - diversity of ischemic and infected-ischemic wounds. Box – plots show the bacterial diversity within the samples as measured using Chao1 (a) and Shannon (b) indices from stages C and D, respectively.

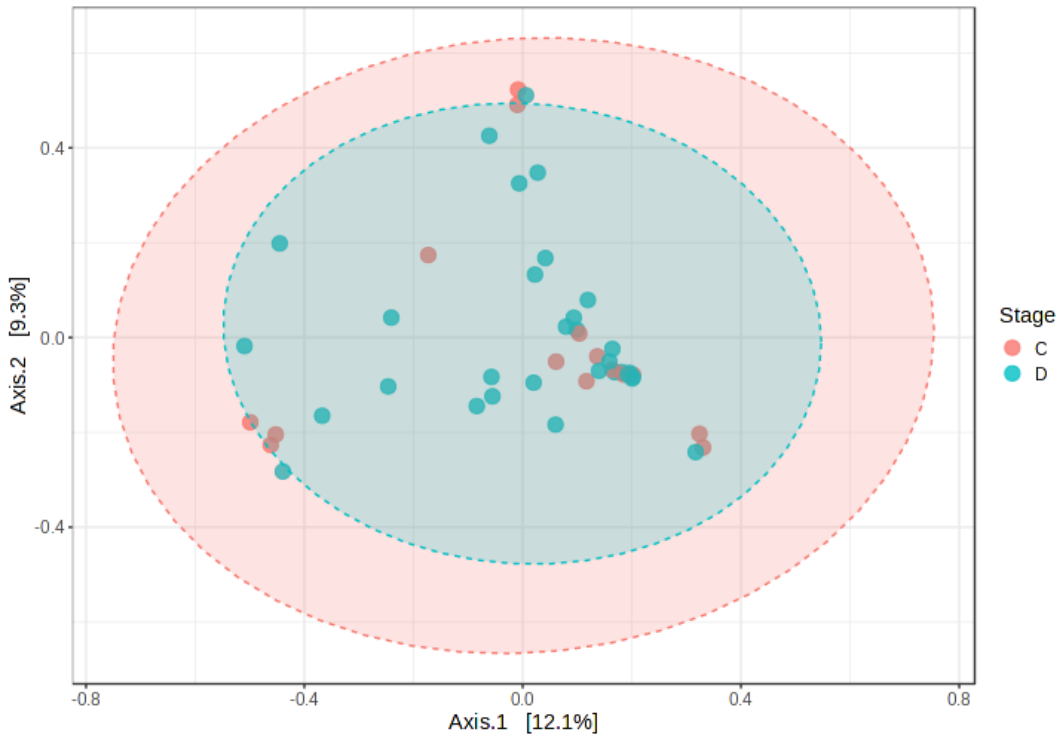
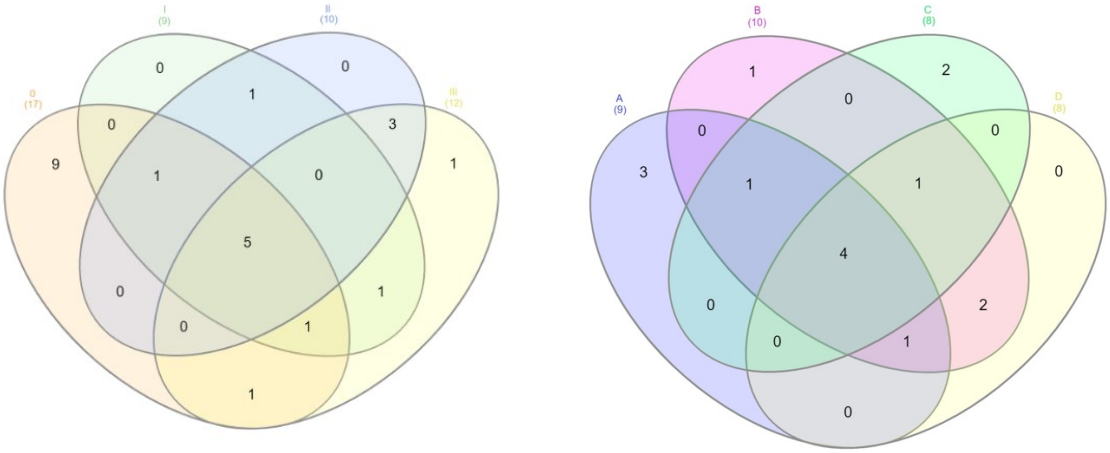


Figure 3.22 Beta - diversity of ischemic and infected-ischemic wounds. PCoA plots with Bray – Curtis dissimilarity does not show a significant difference between stages C and D.

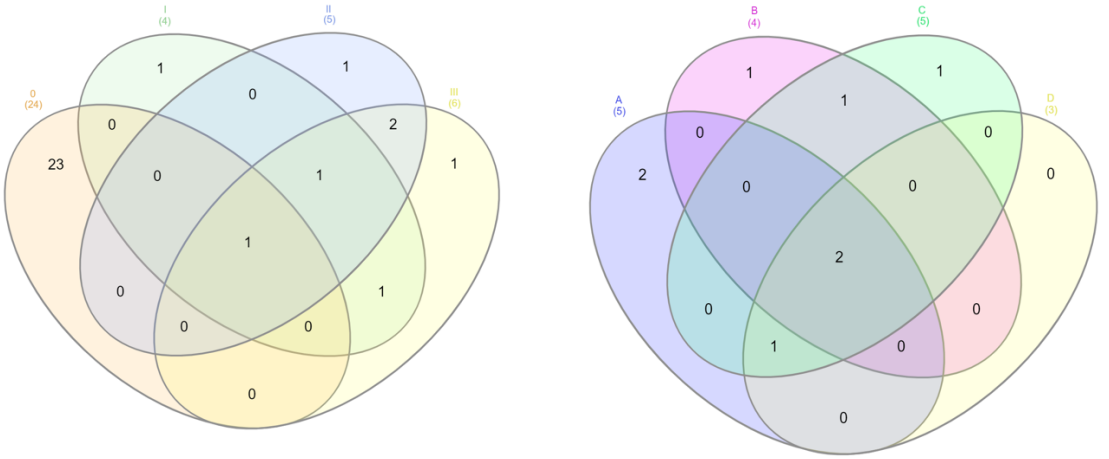
3.4.8 Relationship between taxa of different grades and stages

To observe if certain taxa were unique or common in wounds of different grades and stages, taxa with RA of ≥1% at 20% prevalence were selected. Venn diagram figures were created using InteractiVenn (Heberle et al., 2015) .At the genus level, *Staphylococcus*, *Corynebacterium*, *Finegoldia* and *Peptoniphilus* were commonly found in all stages of the wound (Figure 3.23). *Pseudomonas* was unique to stage B while *Stenotrophomonas* and *Dermabacter* were found unique to stage C and none was solely found to stage D. At a species level, *Corynebacterium striatum* and *Finegoldia magna* were found common to all stages (Figure 3.24). On the other hand, all the genera common to all the stages were also commonly found among all the grades including *Anaerococcus* (Figure 3.23). More genera were found in grade 0 compared to only one genus in grade III (*Pseudomonas*). At a species level, *C. striatum* and *F. magna* were common between all the stages while only *F. magna* was common between all grades (Figure 3.24). In infected wounds (stage B), *P. aeruginosa* was the only species unique to this stage. *S. aureus* was common in stage A, C and D.



(a) (b)

Figure 3.23 Unique and shared taxa (genus) between wounds of different grades and stages. Venn- diagram represents the taxa that were common between grades (a) and stages (b) at a relative abundance of ≥ 1% and 20% prevalence.



(a) (b)

Figure 3.24 Unique and shared taxa (species) between wounds of different grades and stages. Venn – diagram represents the taxa that were common between grades (a) and stages (b) at a relative abundance of ≥ 1% and 20% prevalence.

In an attempt to investigate for potential interaction between *Candida* species and bacteria in DFU, the top 50 most common bacteria species that occur at more than 1% RA in at least two samples grown with *Candida* species were analysed and shown in Figure 3.25.

F. magna (RA range from 1 – 64%) was the only bacteria that was common to all *Candida* species isolated in this study. *C. albicans* were found to be commonly associated with *A. faecalis*, *A. haemolyticum*, *K. aerogenes*, *P. melaninogenica* and *S. oralis*, while only *E. faecium* was commonly found with *C. glabrata*. A more diverse species was observed with *C. parapsilosis* mainly Coagulase-negative *Staphylococcus*.

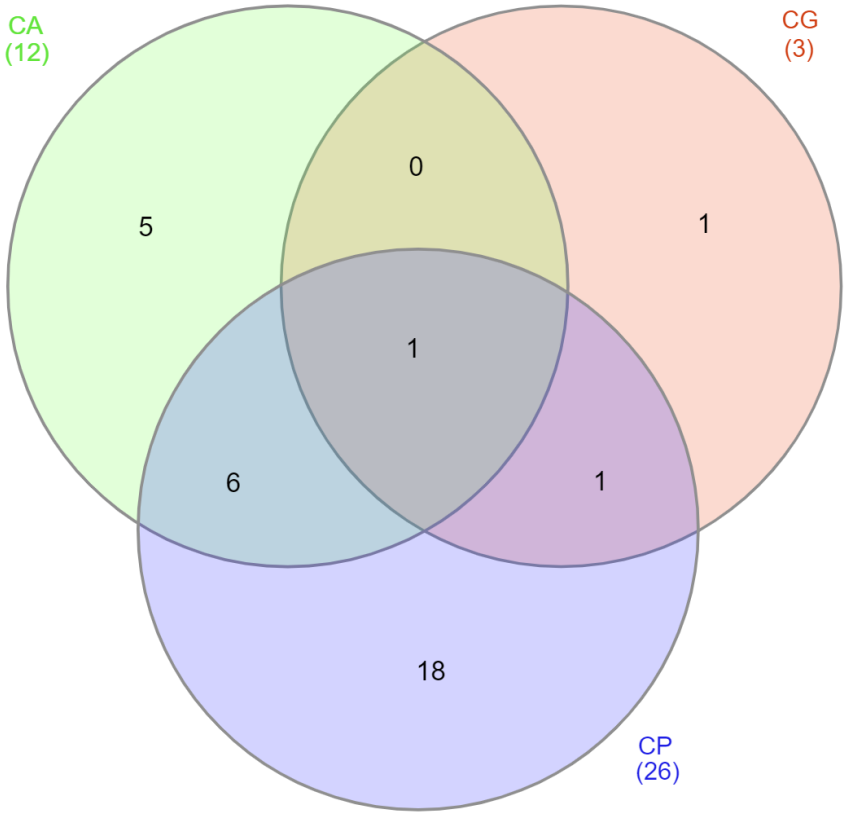


Figure 3.25 Unique and shared taxa between *Candida* species. Venn- diagram represents the taxa that were found at ≥1% relative abundance in at least two samples grown with *Candida* species.

3.5 Discussions

DFU infection is often the cause of delayed healing due to poor response to antibiotic treatments in diabetic wounds (Baig et al., 2022). Evidence suggests that the prevalence of biofilms ranges from 60 – 80% in chronic wounds (James et al., 2008; Malone, Bjarnsholt, et al., 2017; Pouget et al., 2020) and several studies have demonstrated a diverse microbiome in infected DFU (Jneid et al., 2017; Moon et al., 2021). DFU can remain uninfected or exacerbated to a more severe condition involving different tissues including tendons or bone (Cavanagh et al., 2005; Malhotra et al., 2014). To date, very little attention has been paid to comparing microbiome composition covering different depths and severity of the wound using the University of Texas wound classification system. In this chapter, microbiomes were not found to be distinctly clustered despite the stratification of the wounds according to the different grades and stages, but in fact displayed heterogeneity between samples.

Corynebacterium, *Staphylococcus* and *Streptococcus* were the most commonly observed in this study. There are similarities between the most common taxa identified here and as described by previous studies (L. R. Kalan et al., 2019; Loesche et al., 2017; Sadeghpour Heravi et al., 2019). Although the order of abundances may be different between studies, but their abundances are among the highest as reported in each respective study. These studies have also used swabs with samples analysed from multiple healing outcomes including healed and amputated wounds (L. R. Kalan et al., 2019; Loesche et al., 2017). The prevalence of gram-positive bacteria in this present study and also from the previous studies are in agreement with empirical antimicrobial guidance for diabetic foot ulcers that often cover for *Staphylococcus* and *Streptococcus* infections (Lipsky, 2004; Lipsky, Berendt, et al., 2012; Senneville et al., 2024). These antibiotics include Cloxacillin, Clindamycin and Fluoroquinolone amongst others (Senneville et al., 2024). However, the role played by *Corynebacterium* in causing infection is still not clear and subjected to case by case discussion. Empirical guidelines do not explicitly mention them although certain antibiotics such as Vancomycin and Linezolid are effective (Alefiya et al., 2018; Lipsky, Berendt, et al., 2012). Nevertheless, individual susceptibility testing is crucial as species variation could result in different susceptibility profiles (Alefiya et al., 2018). *Corynebacterium* is often considered a contaminant and is usually implicated when samples are

collected from the deep site of infection such as from intra-operative samples. In a study by Bessman et al (1992), the isolation of *Corynebacterium* from the tissue collected during the intra-operative procedure has suggested the pathogenic role of the organism instead of contaminants (Bessman et al., 1992).

The observation of taxa based on genus may not necessarily indicate the pathogenic role played by these taxa as some species were found to be more virulent than others of the same genus and they could interact differently with other bacteria. Several studies have revealed the protective role provided by skin commensal of *S. epidermidis* and *S. lugdunensis* against the pathogenic species of *S. aureus* by the production of inhibitory molecules of serine protease Esp and lugdunin, respectively (Iwase et al., 2010; Zipperer et al., 2016). Moreover, the production of Esp by *S. epidermidis* has been shown to inhibit and eliminate the biofilms formed by *S. aureus* (Iwase et al., 2010). In this study, speciation resulted in the pathogenic bacteria becoming apparent from wounds, as can be seen from the detection of *S. aureus* and *P. aeruginosa* among the most abundant species. These two species are not uncommon in both acute and chronic wound infections. However, *C. striatum* was greatly more abundant than these two pathogenic species, suggesting that chronicity of the wound may not be driven solely by the pathogenic species. In a study by Ramsey et al., the co-existence of *C. striatum* and *S. aureus* has been shown to turn down the expression of virulence in *S. aureus* to become into commensal state (Ramsey et al., 2016).

Furthermore, *C. striatum* was considered a common coloniser of the skin but may also become an opportunistic pathogen in certain conditions (Flowers & Grice, 2020; Martínez-Martínez et al., 1997). Here, *C. striatum* was also found to be more abundant in non-infected than infected superficial wounds. As some samples were subsequently collected over time (indicating a non-healing state of the wound), intuitively it may seem possible that a reduction of the species might lead to the wound becoming infected. Similarly, a study by Min et al (2020) has shown a reduced abundance of *Corynebacterium* genera in non-healing wounds (Min et al., 2020). The growing concern about the multi-drug resistance of the species might also explain the prevalence of the species identified from various clinical samples (Flowers & Grice, 2020; Otsuka et al., 2006). However, the resistance profiles of the species have not been investigated here and beyond the scope of this present

study. Nevertheless, by comparing the pooled samples together, the exact repercussions of their reduction or displacement by other taxa were not clear. Therefore, single patient analysis on a series of samples might help to better understand the temporal changes of the microbiome as the wounds progress.

Furthermore, the sequencing method has greatly improved the detection of anaerobes from the clinical samples compared to the culture-based method as similarly described from previous studies (Dowd, Wolcott, et al., 2008; Mudrik-Zohar et al., 2022; K. Smith et al., 2016). These anaerobes include *Peptoniphilus*, *Anaerococcus*, *Finegoldia*, *Porphorymonas*, *Prevotella* and *Bacteroides*. In this study, the most common anaerobes include *Peptoniphilus*, *Prevotella*, *Porphorymonas* and *Finegoldia* revealing the proximity of these taxa with other commonly identified taxa usually recovered from culture including *Staphylococcus*, *Streptococcus*, *Pseudomonas* and Enterics bacteria. Dowd et al (2008) proposed the concept that grouped the bacteria based on co-occurrence known as Functionally equivalent pathogroups (FEP), and found that anaerobes contributed to all the identified FEP from chronic DFU samples (Dowd, Wolcott, et al., 2008). In addition, one study has seen a greater abundance of Gram-positive anaerobic cocci contributed mainly by *Peptoniphilus*, *Finegoldia*, *Anaerococcus* and *Helcococcus* in non-healing wounds and has suggested that *Peptoniphilus* could be a marker to anticipate the prognosis of DFU healing (Min et al., 2020).

Moreover, *F. magna* was the most abundant species of obligate anaerobes found in this study and was the species that was found in all stages of the wounds besides *C. striatum*. This may not be surprising as *F. magna* previously known as *Peptostreptococcus magnus*, has been reported in noninfected, chronic and acute wounds since several decades ago (Bowler et al., 2001). Similar to *C. striatum*, *F. magna* was also found as a common coloniser of the skin although some studies have reported serious cases of infected wounds involving amputations (Scapaticci et al., 2018). An adhesin factor (FAF) expressed by most *F. magna* strains was one of the virulence factors that were associated with attachment, and tolerance to host immunity and is also thought to prevent wound healing (Boyanova et al., 2016). Biofilms formed by *F. magna* are among the strongest and comparable to other anaerobes tested including *Clostridium* and *Bacteroides* species (Donelli et al., 2012). However, the study is only limited to biofilm characterisation among

the anaerobes. Therefore, their presence should be considered important when managing chronic wounds. Moreover, their prevalence in various wound conditions may suggest a diverse spectrum of interactions that could occur with other taxa although this remains to be investigated.

Another important finding is the abundance of *A. haemolyticum* as one of the most common species found in this study when all the samples were analysed together. One possible explanation is caused by the recurrence of this taxa in multiple visits and the increase in sample size compared to when only a single visit was analysed. This finding revealed the inadequacy of a single sample when studying microbiome, therefore limiting the finding of rare and infrequent taxa. Although *A. haemolyticum* often reported as a cause of pharyngitis and respiratory infection (Alrwashdeh et al., 2023; Mackenzie et al., 1995), few cases were reported from wound infection(Ceilley, 1977; Choi et al., 2012; Malini et al., 2008; Moon et al., 2021; Thomas et al., 2022). Often, the infection with *A. haemolyticum* was polymicrobial and has been associated with bacteria including *Streptococcus* species, *Proteus* species and *S. aureus* (Choi et al., 2012; Malini et al., 2008; Thomas et al., 2022). A study by Moon et al. has shown that *A. haemolyticum* is the species that failed to be detected by culture but was detectable by 16S sequencing (Moon et al., 2021). An outbreak of *A. haemolyticum* has been reported in the Netherlands in patients with chronic wounds (Bruins et al., 2020). In addition, biofilm formation has been reported in one study of diphtheroids including *A. haemolyticum* (previously known as *C. haemolyticum*) isolated from the wound, but with no distinction between individual capacity of every species (Chandran et al., 2016).

From the data of the previous chapter, DFU does not only contain bacteria but also fungi. Interaction between bacteria and fungi should not be overlooked because previous studies have proven the mutual interaction between these two kingdoms mostly from studies of gut and oral microbiome (H. Li et al., 2022). A more diverse species of bacteria was able to be detected with 16S sequencing. This includes obligate anaerobe (*F. magna*) that was commonly found with all the *Candida* species in this study. Many studies have revealed some form of interaction between common pathogens including *S. aureus*, *P. aeruginosa* and *E. faecalis* but none have investigated the interaction between *F. magna*. It was not surprising to

have seen many studies describing the good and bad interaction between *Candida* and *Enterococcus* as they were also seen to be common in this study although their interaction suggesting a species-specific interaction, with *C. albicans* and *C. glabrata* were more commonly found with *E. faecalis* and *E. faecium*, respectively. *In vitro* studies have very well demonstrated the complexity of interaction between *E. faecalis* on *C. albicans* (Alshanta et al., 2022), but none has been studied on *E. faecium* and *C. glabrata* interaction.

There was a more diverse set of taxa that were uniquely associated with *C. parapsilosis* than *C. albicans*. The taxa associated with *C. parapsilosis* are usually found on the skin such as Coagulase-negative *Staphylococcus* (CoNS) and from the environment. On the other hand, *C. albicans* tend to be more associated with bacteria from gastrointestinal tract (*A. faecalis*, *A. haemolyticum*, *K. aerogenes*, *P. melaninogenica* and *S. oralis*). This pattern may not be surprising as *C. albicans* is found around 80% in the gut of healthy individuals (Delavy et al., 2023) while *C. parapsilosis* is a common coloniser of the skin (E. M. da Silva et al., 2021). Thus, it seems possible they have a set of preferences to maintain that interaction in order to thrive. This niche-related co-existence reflects the tendency for the species to maintain the innate interaction to function outside their normal ecological niche. Perhaps disrupting this coalition may lead to dysbiosis of the microbiome leading to clearance by the host immune response as a previous study had shown that the less stable the wound microbiome, the better the outcome (Loesche et al., 2017). Unlike certain diseases like irritable bowel disease, dysbiosis is associated with the disease (DeGruttola et al., 2016). Nevertheless, both fungi were found to be commonly associated with pathogenic species such as *S. aureus* and *P. aeruginosa*.

In this study, the diversity of wound microbiomes was found to be similar when compared across the grades and stages. However, there were few studies had reported different findings. Gardner et al. found an increase in bacterial diversity as the wound depth increases with more anaerobes identified (S. Gardner et al., 2013). However, two studies that guided by Wagner grading system have reported contradictory findings. Jnana et al. have found a more diverse bacterial population in the highest wound grades (WGS5), but Park et al. have not found a difference between low and high grades (Jnana et al., 2020; Park et al., 2019).

When compared between skin and wound, the latter was found to be less diverse (Park et al., 2019). Regardless of the indices measured, wounds were found to be polymicrobial.

Several studies have also shown the correlation between microbial load and wound healing by implicating high bacterial load with delayed wound healing (Chong et al., 2020; Soldevila-Boixader et al., 2022). These findings were based on the total count of the CFU grew in culture media. However, the bacterial load measured in this current study were similar across different depths and severity of the wounds. This finding supports the evidence from previous work by Demetriou et al. who also observed that ulcer severity and duration do not correlate with the increase in the microbial load (Demetriou et al., 2013). Moreover, the microbial load in this study was also in a similar range (~ 10^4 - 10^9) as reported by Armstrong and colleagues (2023), although the study used fluorescent imaging to measure the bacterial load directly on the DFU (Armstrong et al., 2023).

The DFU microbiome is certainly polymicrobial consisting of bacteria and fungi from the skin, GI tract as well as patients specific surrounding environment. Since the samples collected in this study do not conform to a rigid experimental design, the microbiome described in this study should reflect a real-life snapshot of the DFU microbiome. While the overall microbiome picture may help in empirically guiding and managing the wound, we must be mindful that the DFU microbiome is patient-specific. Therefore, the precision of treatment may be achieved by understanding the temporal changes of the microbiome over time.

Chapter highlights:

DFU microbiomes are polymicrobial and heterogenous among patients.

Severity and wound depth are not associated with a distinct population of bacteria.

4 The dynamic of DFU microbiomes: Patients' journey

4.1 Introduction

Understanding causative microbes in a particular infection is a major pre-requisite shaping the treatment guidelines facilitated by existing diagnostic approaches in identifying those pathogens. A diverse range of microbes may include bacteria of various origins, including skin, gut, oral and the surrounding environments (Malone, Johani, et al., 2017). Over the last few years, the options of empirical antibiotics in managing DFU have remained unchanged, however, some additional antibiotic agents of different classes have been added in recent years (Lipsky et al., 2020; Senneville et al., 2024). In addition to the traditional antimicrobial treatment, novel strategies, including the use of cold plasma and newly discovered drug delivery methods, have shown some promise (Barjasteh et al., 2023; M. Liu et al., 2023). Nevertheless, chronic DFU is still an ongoing burden to the healthcare system (Waibel et al., 2024).

Previous studies have focused on establishing a universal picture of the wound microbiome associated with various experimental factors, including clinical inflammatory markers, wound severity and healing stage (S. Gardner et al., 2013; Jnana et al., 2020; MacDonald et al., 2019). The generalisability of these studies has overlooked the distinctive and unique microbiome that every individual wound would possess. Moreover, the nature of comparing microbiome data is traditionally based on the most common and abundant taxa in a particular cohort, resulting in the underrepresentation of certain species. Furthermore, pooling analyses of the sequencing data from all samples will only highlight the major species across the study cohort. While this is useful for addressing the universal idea of the wound microbiome, precise polymicrobial interactions within the wound may only be deciphered when looking at individual wound journeys over time (Nahid et al., 2021).

To understand the behaviour of the DFU microbiome, it is crucial to observe and consider the baseline microbiome as the wound progresses sequentially (Gardiner et al., 2017; Loesche, 2016). Much uncertainty still exists about the unique biological signature of the wound microbiome and interactions between different species of bacteria in this environment. It is impossible to precisely elucidate this relationship in association with a particular clinical pathology by only looking at the microbiota at a single time point within a wound. Longitudinal profiling of

individual wound microbiomes will generate an improved insight into the temporal changes that happen as the wound progresses.

4.2 Hypothesis and aims

Patient specific microbiome profiles underpin the chronicity and morbidity of the wound, and result in differential responses towards antibiotic therapies. While traditional treatment targets the most likely pathogenic species in the wound, elimination of one species could lead to the growth of other species (C. Liu et al., 2020). Therefore, it is hypothesised that the chronic wound microbiome constitutes a unique polymicrobial interaction that continuously adapts in response to treatment and the host.

This chapter will aim to demonstrate the compositional dynamics of wound microbiomes from patients with multiple visits and explore their responsiveness to therapeutic interventions.

4.3 Materials and methods

4.3.1 Sample selection

This chapter takes an alternative analysis approach to the wound microbiome data by focussing on individual patients' journeys. The analyses presented in this chapter were based on the data that have been obtained and processed in a previous chapter (Chapter 3). Ten patients with multiple visits were selected for longitudinal analysis of the microbiomes in this chapter. Samples of interest are from patients 2, 10, 18, 20, 93, 97, 99, 111, 117 and 123. Data on prescribed antibiotics for all these patients were obtained and recorded by the clinical research teams from Royal Lancaster Infirmary.

4.3.2 Statistical analysis

Microbiome analysis was performed using MicrobiomeAnalyst 2.0 (Chong et al., 2020) and figures were created using GraphPad Prism. The sequencing reads for every group of the selected patients were rarefied according to the lowest reads of the sample in the group before analysis was performed.

4.4 Results

4.4.1 Samples demographic

Clinical samples, as previously described in Chapter 2, were based on grades and stages and grouped together by patient for longitudinal analyses. A total of 80 samples were analysed, with the number of visits ranging from 4 to 13 visits across 22-86 weeks (depending on the individual patients). All patients were prescribed with antimicrobials of various groups as presented in Table 4.1.

Table 4.1 Samples demographic with history of antibiotics

Patient	Number of visits	Follow- up period (Weeks)	Prescribed antibiotics/antifungals
2	8	74	Clarithromycin, Co-amoxicillin, Flucloxacillin, and Fluconazole
10	11	86	Clindamycin, Ciprofloxacin, Linezolid, Clarithromycin, Flucloxacillin
18	7	75	Clindamycin, Ciprofloxacin, Doxycycline, Amoxicillin, Co-amoxicillin, Flucloxacillin, Clarithromycin, Gentamicin
20	4	85	Flucloxacillin, Co-amoxicillin
93	13	56	Co-amoxicillin, Flucloxacillin, Doxycycline, Clindamycin, Ciprofloxacin, Clarithromycin
97	5	25	Clindamycin, Metronidazole, Teicoplanin
99	6	53	Clindamycin, Co-amoxicillin, Doxycycline, Levofloxacin
111	6	22	Co-amoxicillin, Clindamycin, Flucloxacillin
117	6	34	Co-amoxicillin, Flucloxacillin, Clindamycin, Doxycycline
123	4	30	Clindamycin

4.4.2 Case reports - individual patient bacterial composition and diversity

To observe the dynamics of microbiome composition for each patient, the relative abundance of bacterial species was visualised against the timeline of follow-up visits. It was not possible to control the frequency of sample collection for every patient, as these were collected according to the patient's condition. Therefore, the period between visits varies across patients. Furthermore, some patients may present with multiple wounds that differ in microbiome composition, and therefore each visit the microbiome may not necessarily be indicative of the same wound. The location of the wound was indicated in the plot when involved multiple wounds.

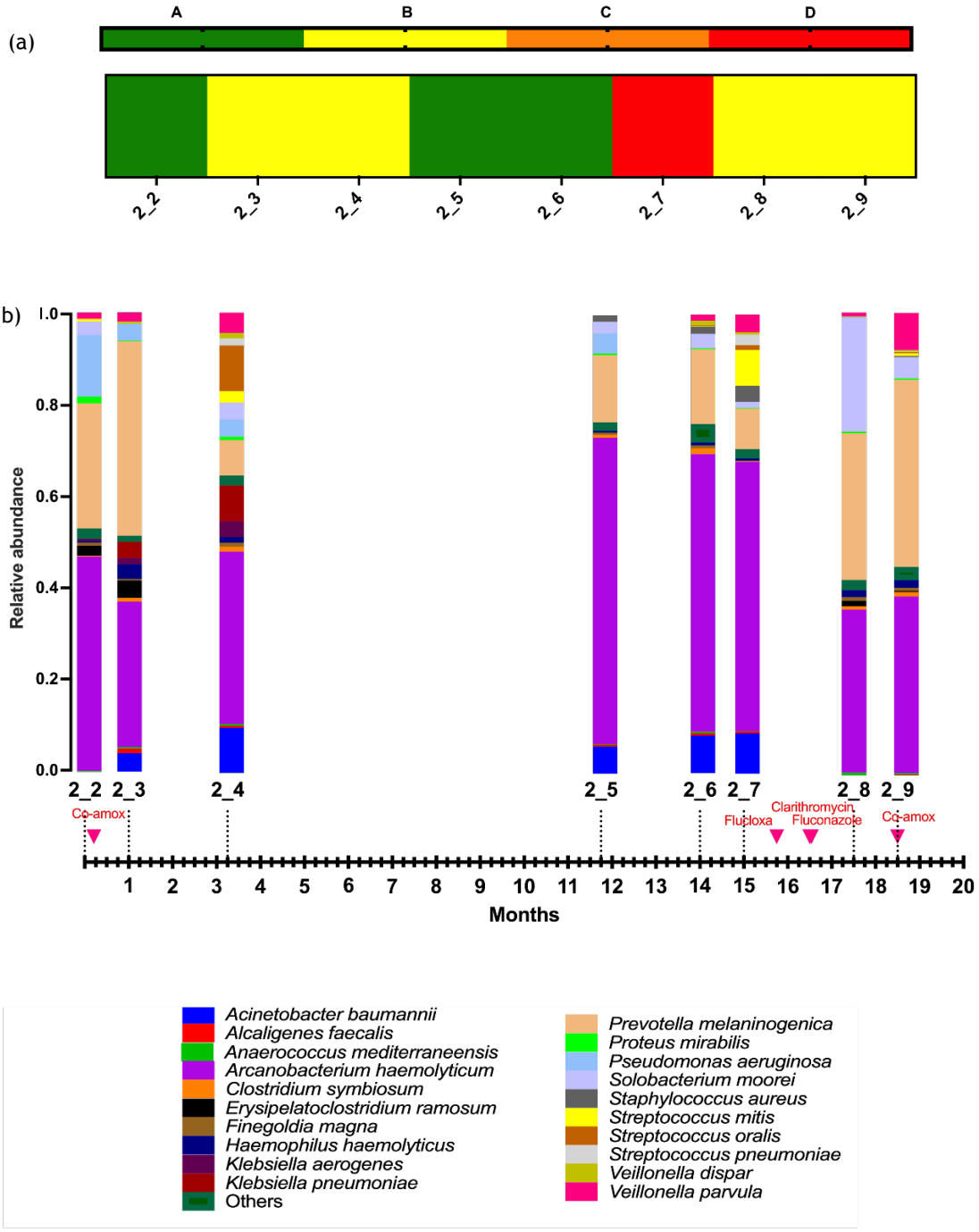
Patient 2

Patient 2 presented with a single wound located on the right foot which persisted for 74 weeks. Based on their records, the patient had undergone amputation (June 2018) before the start of this study, therefore the wound indicates a recurrent ulcer. During follow-up, the wound condition mostly remained static with exacerbation noted during visit 2_7. The microbiota across all the visits shows that the wound consisted of two predominant species of bacteria (*Arcanobacterium haemolyticum* and *Prevotella melaninogenica*). These species were prevalent in the patient's wound throughout the study period (Figure 4.1). The presence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were other important and common pathogens in this patient. It was noted that their presence was not concurrent, except in visit 2_5 where both were detected. *P. aeruginosa* was initially present but not detected when *S. aureus* appeared after the co-detection of both species in visit 2_5.

The diversity as measured by Chao1 and Shannon's diversity index revealed a fluctuation over time with the highest diversity measured in visit 2_4 with a sharp decline in the next visit (Figure 4.1). There was an average of 24 observed species as measured by Chao1 estimate across all the visits. In contrast, standard culture shows four major groups of bacteria including mixed skin, enterics, coliforms and anaerobes. *S. aureus* was isolated in all visits except in the sample collected during visit 2_2. This finding does not match the microbiome data where *S. aureus*

was isolated in almost every sample. Moreover, standard culture does not capture the presence of *P. aeruginosa* as shown by the sequencing data. The presence of anaerobes was not consistently isolated in all the samples. Interestingly, apart from bacteria, *C. albicans* was isolated from samples 2_2-2_6.

During this study, antibiotics and antifungals were mainly prescribed from visit 2_7-2_9. Initial treatment was with Co-amoxicillin, a broad-spectrum antibiotic given after visit 2_2. Despite the isolation of *S. aureus* from standard culture starting at visit 2_3, the patient was only treated with Flucloxacillin at visit 2_7 when the wound condition had worsened. Clarithromycin and Fluconazole were also added to the treatment. However, microbiome compositions were comparable between the first and last visit with *A. haemolyticum* and *P. melaninogenica* remaining present and dominant. The patient was treated with Co-amoxicillin with the persistent isolation of *S. aureus*, coliforms, and anaerobes. The wound remained unhealed at the end of the study period.


(a)
A
B
C
D
2_2
2_3
2_4
2_5
2_6
2_7
2_8
2_9
(b)
Relative abundance
1.0
0.8
0.6
0.4
0.2
0.0
2_2
2_3
2_4
2_5
2_6
2_7
2_8
2_9
Co-amox
Clarithromycin
Flucloxa
Fluconazole
Co-amox
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Months
Acinetobacter baumannii
Alcaligenes faecalis
Anaerococcus mediterraneensis
Arcanobacterium haemolyticum
Clostridium symbiosum
Erysipelatoclostridium ramosum
Finegoldia magna
Haemophilus haemolyticus
Klebsiella aerogenes
Klebsiella pneumoniae
Others
Prevotella melaninogenica
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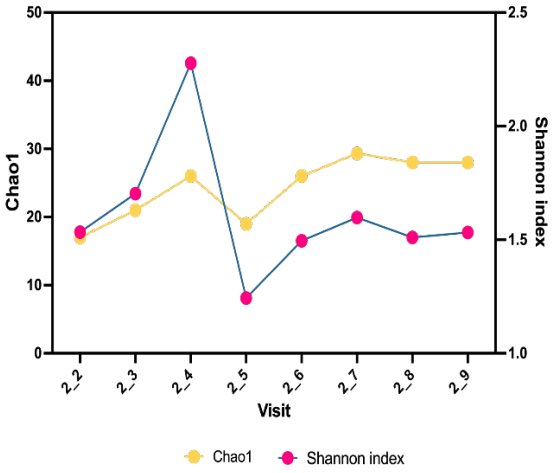
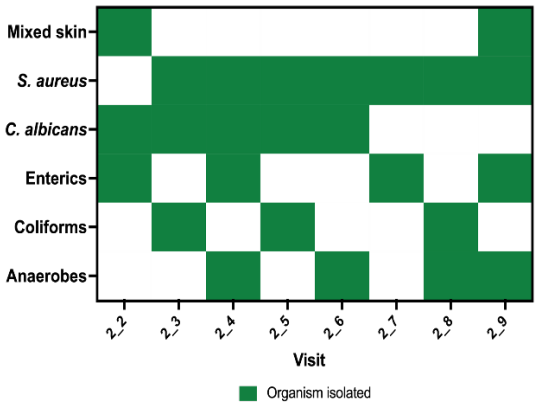
Streptococcus pneumoniae
Veillonella dispar
Veillonella parvula



(c)



(d)

Figure 4.1 Timeline and microbiome profile of patient 2. (a) Stage of infection for each visit. (b) Relative abundance of bacterial species against the visit timeline with antibiotics treatment denoted with pink triangle. (c) α-diversity as measured by Chao1 and Shannon index. (d) Organisms isolated coloured in green as obtained from standard microbiology culture.

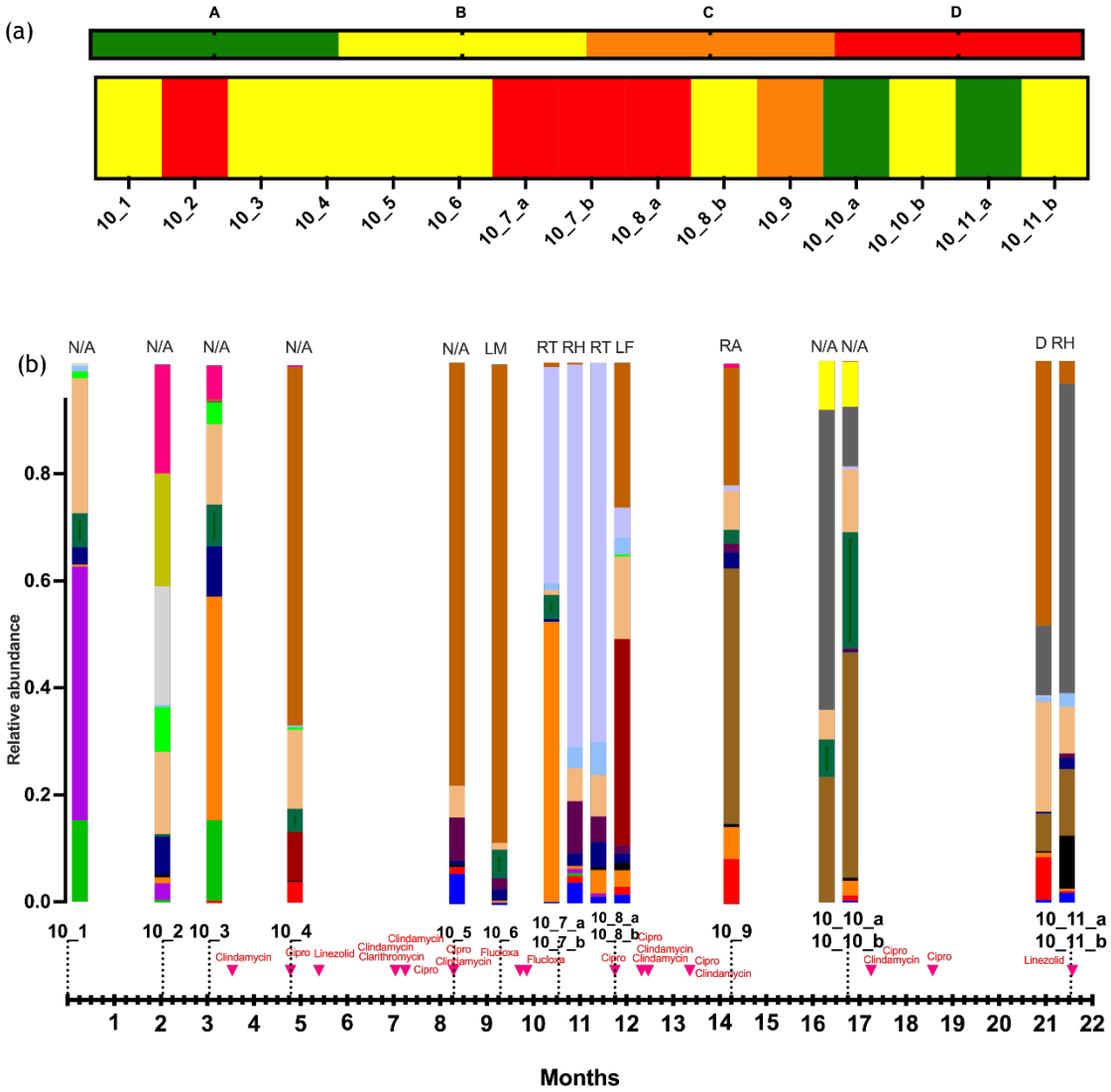
Patient 10

Patient 10 presented with multiple wounds located on both feet and was followed up for 86 weeks (Figure 4.2). Unfortunately, due to incomplete metadata for patient 10, wound locations during the 5 initial visits were not noted. Sample 10_6 was the only sample collected from the left medial foot and healed during the follow-up period. The wound consisted predominantly of enteric bacteria including *Serratia marcescens* and *Klebsiella pneumoniae*. Sample 10_7_a and 10_7_b were collected from the right toes and right heel, respectively. There was an overlap in the species that were present in both locations. This includes *Porphyromonas bennonis* which comprised 40% (right toes) and 70.7% (right heel) of the bacterial community at each site. *Corynebacterium striatum* was also present in high abundance in the right toes. Follow-up of the right toes shows the persistence of the *P. bennonis* that accounted for the most abundance species but with decreasing abundance of *C. striatum*. The wound eventually underwent amputation and the post-amputation microbiome at 10_9 shows predominance of enteric bacteria such as *Escherichia coli* and *S. marcescens*. The alpha diversity of the right toe wound showed a decline post-amputation as measured by Chao1(22 to 19), but an increase with Shannon index (1.33-1.76).The wound condition improved while wounds at the last follow-up remained colonised by enteric bacteria. Overlapping of the species from wounds located on the same foot can also be seen in samples 10_11_a and 10_11_b, which were collected from the dorsum and heel of the right foot, respectively. Both samples consisted of *Porphyromonas somerae*, *S. marcescens* and *E. coli*. There were 24 average species observed in this patient across all samples.

When compared to standard culture, the first two visits showed the presence of *S. aureus* and *Candida* species. In visits 3-5, wounds were mainly isolated with coliforms bacteria, co-isolated with anaerobes and *Candida* species at visits 3 and 4, respectively. In visit 5, coliforms were isolated with mixed skin. However, as mentioned earlier, the wound's location was not specified. The wound from visit 10_6 showed the isolation of coliforms. Wounds of visit 10_7_a and 10_7_b showed only the presence of coliforms and enterics bacteria, respectively. Subsequent sampling from the right toe showed the persistence of coliforms in the wound. Culture data from 10_10_a and 10_10_b were not available from the record. Wound from dorsum (10_11_a) was isolated with enterics, while right heel of the

same foot (10_11_b) showed a mixed growth of skin microbiota, coliforms and *Candida* species.

Clindamycin and Ciprofloxacin were the most common antibiotics prescribed for this patient. Initially, Clindamycin was prescribed for the patient after visit 10_3. Multiple antibiotics were prescribed between visits 10_4 and 10_5, including Ciprofloxacin, Linezolid, Clarithromycin, and Clindamycin. Repeated treatment with Ciprofloxacin and Clindamycin can be observed between visit 10_8 and 10_11. Besides broad-spectrum antibiotics, Flucloxacillin has also been used between visits 10_6 and 10_7.



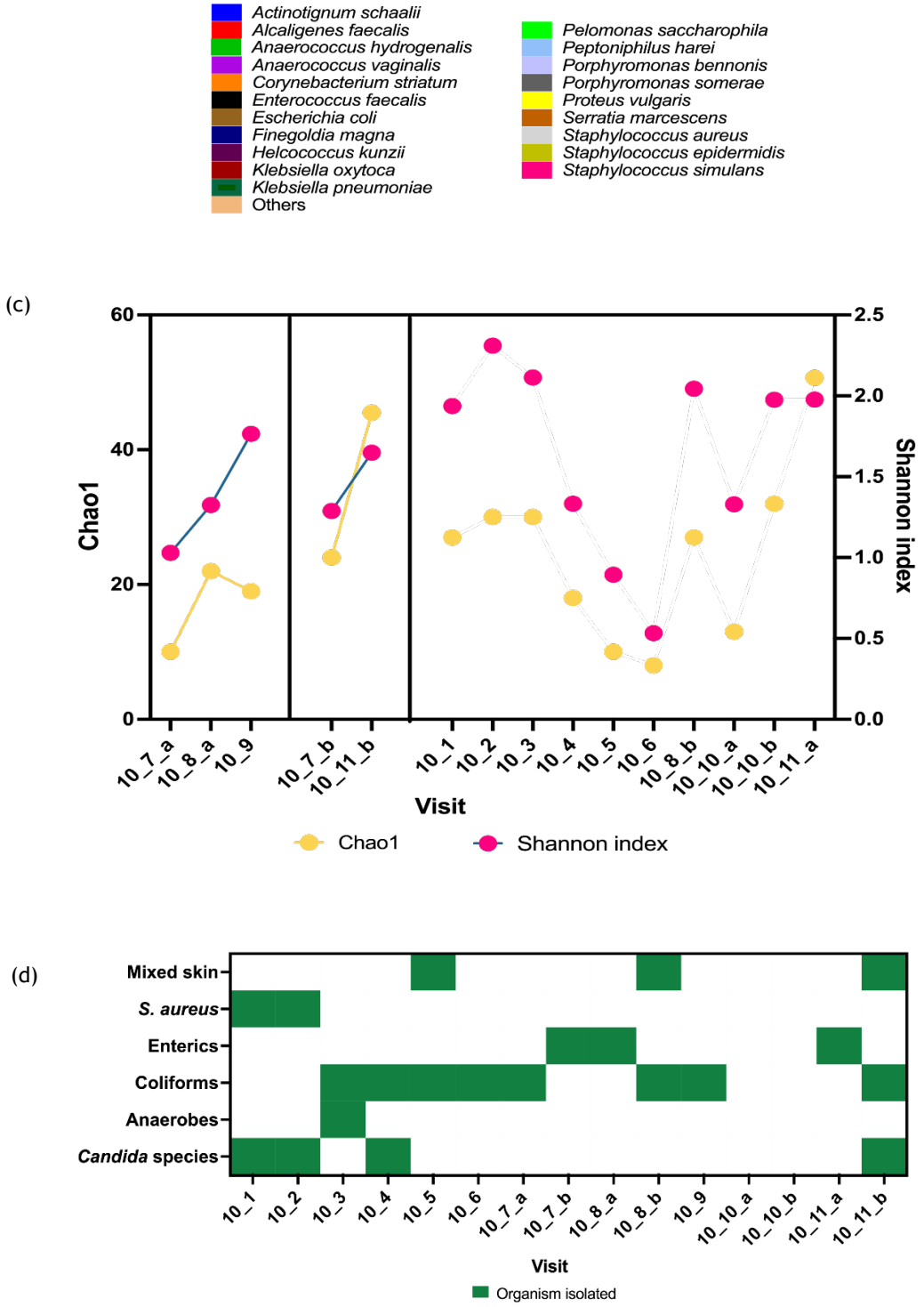
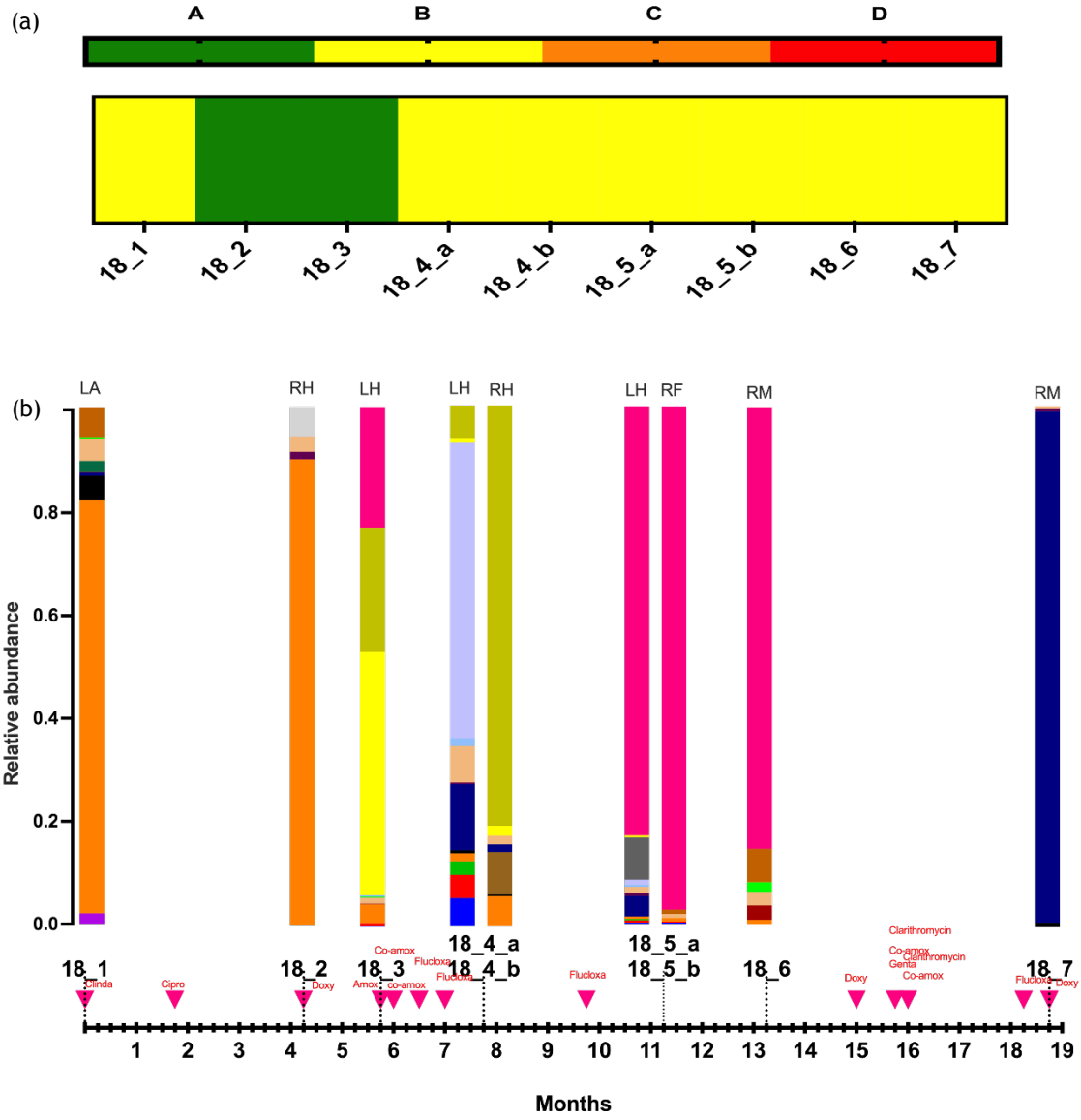


Figure 4.2 Timeline and microbiome profile of patient 10. (a) Stage of infection for each visit. (b) Relative abundance of bacterial species against the visit timeline with antibiotics treatment denoted with pink triangle. Distinct locations of the wound were denoted as left medial (LM), right toes (RT), right heel (RH), left forefoot (LF), Right amputation site (RA), Dorsum (D), locations not available (N/A) (c) α-diversity as measured by Chao1 and Shannon index (d) Organisms isolated coloured in green as obtained from standard microbiology culture.

Patient 18

Patient 18 presented with multiple wounds from both feet across 75 weeks. These include the left ankle, left heel, right heel and right metatarsophalangeal joints (MTPJ). At the beginning, infected superficial wound was presented on the left ankle. It was apparent from Figure 4.3 that *C. striatum* was the most abundant organism in the wound at 79.8%. *S. aureus* and *Enterococcus faecalis* were also identified at 5.79% and 4.77%, respectively. The patient was treated with Clindamycin and Ciprofloxacin before the second visit. However, during the second visit, only the new wound on the right heel was collected. Similar to the previous wound on the left foot, the predominant species found was *C. striatum* at 89.9%. Six weeks later, a wound from the left heel close to the location of the first wound was taken shown a decline in *C. striatum* to 3.8% with the microbiome primarily consisting of *S. marcescens*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* in descending order of relative abundances. Fluctuation of diversity was observed in the left heel by both Chao1 and Shannon index. Antibiotic treatments including Doxycycline, Co-amoxicillin and Flucloxacillin were prescribed to the patient before the fourth visit at ~8 weeks apart. At the fourth visit, infection involved a deep site (grade II) for both sides of the feet (left and right heel). *S. agalactiae* was found in both wounds with a higher abundance on the right side while the left wound was predominated by *P. bennonis*. *Enterococcus faecium* was another important pathogen that was detected in the right heel. Flucloxacillin was again prescribed, and wounds were followed up at about ~14 weeks after and the right heel wound has healed. On the other hand, the left heel wound remains infected, which is predominated by *S. dysgalactiae*, similar to other wounds collected on the right foot. The subsequent follow-up involved a new wound from the right MTPJ with *S. dysgalactiae* remaining as the dominant species. The return of *S. aureus* was also identified at 6.4% of RA and additional antibiotics were prescribed including Doxycycline, Co-amoxicillin, Gentamicin, Clarithromycin and Flucloxacillin. After ~22 weeks, the right MTPJ wound was sampled again and the microbiome was almost entirely predominated by *E. coli*. The average diversity measured by Chao1 was 13 for all the samples. However, the average diversity was higher on the left compared to the right foot with 19 and 8 of observed taxa, respectively.

Wounds of right heel (18_2 and 18_4_b) showed the persistence of *Candida* species. Besides that, wounds 18_2 and 18_4_b were co-isolated with coliforms and enterics bacteria, respectively. For left heel wounds, initially, the wound was isolated with *Streptococcus* group b and coliforms bacteria (18_3). Subsequent culture showed the elimination of both organisms from the wound, but was replaced by *S. aureus*. In the follow-up culture (18_5_a), a mixed growth of *S. aureus* and *Streptococcus* group G was obtained. The last two visits of wound from right MTPJ showed the persistence of *S. aureus* in the culture.



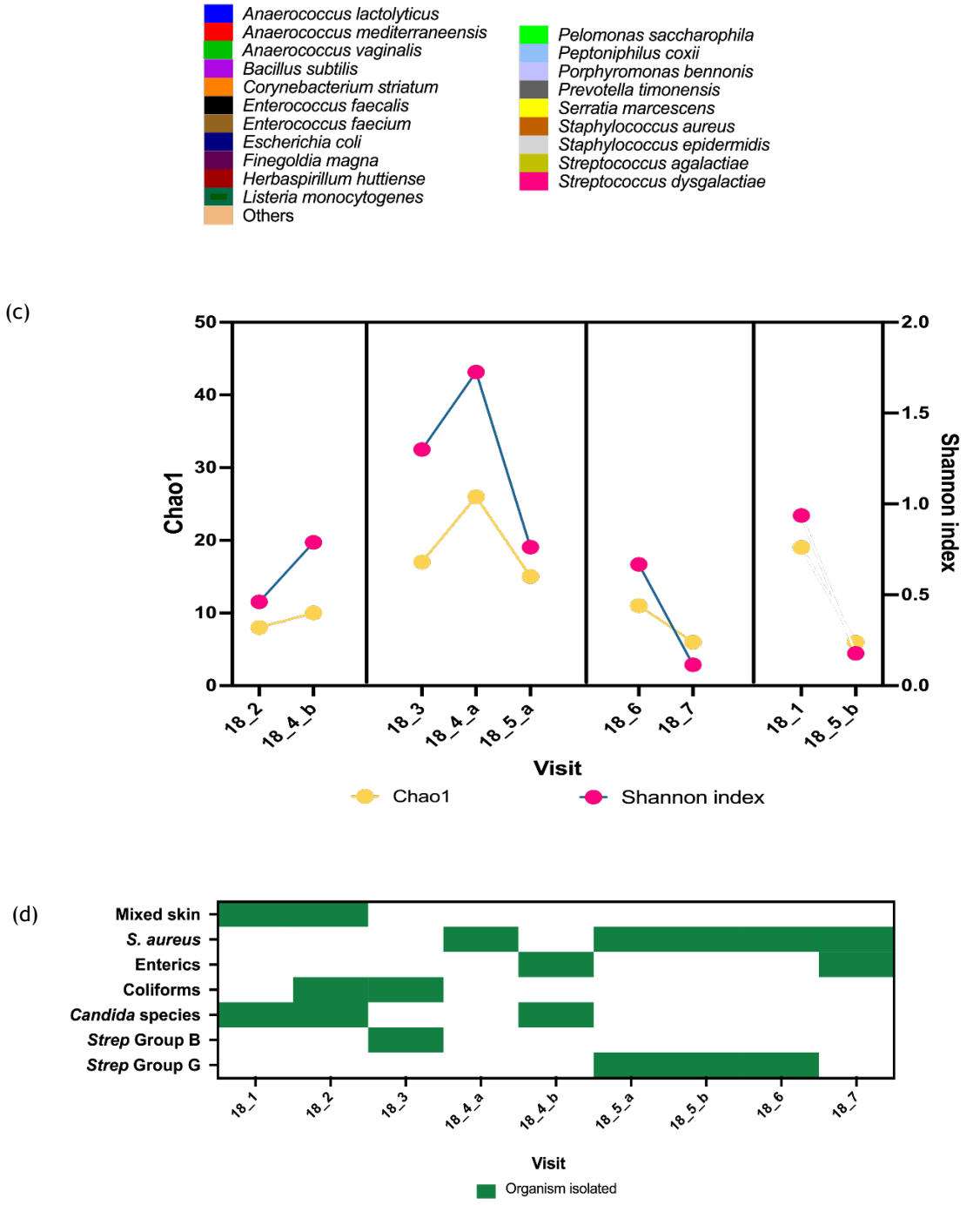
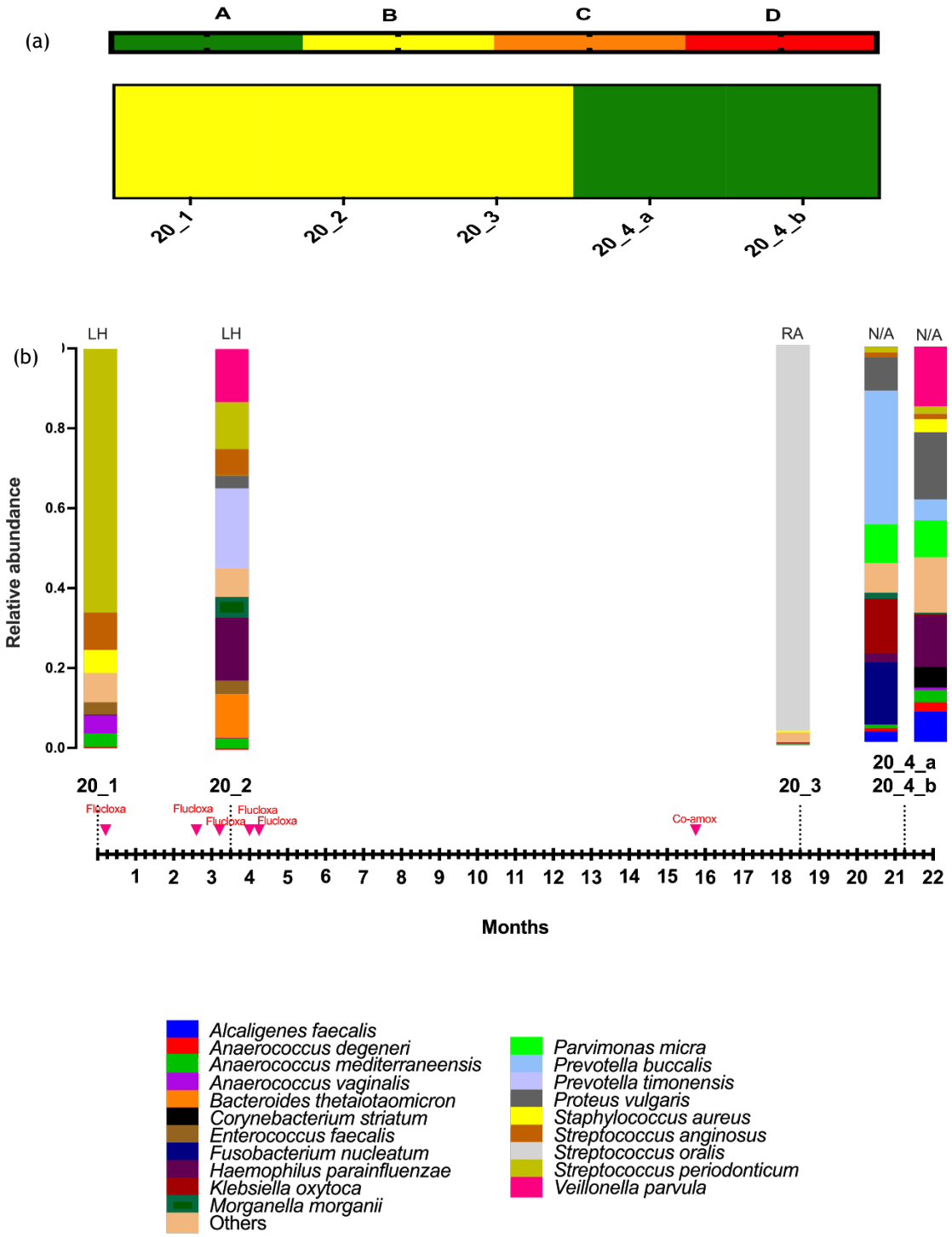


Figure 4.3 Timeline and microbiome profile of patient 18. (a) Stage of infection for each visit. (b) Relative abundance of bacterial species against the visit timeline with antibiotics treatment denoted with pink triangle. Distinct locations of the wound were denoted as left ankle (LA), right heel (RH), left heel (LH), right foot (RF), Right metatarsophalangeal joints (RM) (c) α-diversity as measured by Chao1 and Shannon index. Samples indicate wounds from right heel (18_2 and 18_4_b), left heel (18_3, 18_4_a and 18_5_a), right MTPJ (18_6 and 18_7). Unpaired samples showed by 18_1 and 18_5_b (d) Organisms isolated coloured in green as obtained from standard microbiology culture.

Patient 20

Wounds of patient 20 were collected from left heel and right foot across 85 weeks (Figure 4.4). Initially, the patient presented with an infected left heel wound which shows *Streptococcus periodonticum* (66%) as the most dominant species, with common pathogens including *S. aureus* (5.74%) and *E. faecalis* (3.07%) also present. The patient has also been treated with Flucloxacillin multiple times between the first and second visit. The major taxon which was *S. periodonticum* decreased to 11.68% after about 17 weeks of repeat sampling. While *S. aureus* was still detectable, the abundance was reduced to <1%. On the other hand, the abundance of *E. faecalis* remained similar between the two visits. An increased in diversity was observed between the two visits (Chao1;29 to 39, Shannon; 1.44 to 2.44) .After over a year (~57 weeks), the patient returned with an infected deep wound collected from the amputation site of the right foot. However, there was no sample collected during this study on amputated wounds. The microbiome at this stage was almost entirely composed of *Streptococcus oralis* at 96.4%, with *S. aureus* detected in less than 1%. During visit 4, swabs were collected from both wounds and microbiomes showed some overlaps in microbiota that were present. These include *Prevotella buccalis* and *Proteus vulgaris*. Despite being relatively low in abundance, *S. aureus* was still detected in both samples.

Based on the standard culture, *S. aureus* was persistent in all the collected wound samples. Besides that, the first sample of left heel have also been co-isolated with *Enterococcus* species which then showed no growth in the subsequent culture. The last three visits showed the co-isolation of *S. aureus* and enterics bacteria in the wounds.


(a)
A
B
C
D
20_1
20_2
20_3
20_4_a
20_4_b
(b)
LH
LH
RA
N/A
N/A
Relative abundance
0.0
0.2
0.4
0.6
0.8
20_1
20_2
20_3
20_4_a
20_4_b
Flucloxa
Flucloxa
Flucloxa
Flucloxa
Flucloxa
Co-amox
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Months
Alcaligenes faecalis
Anaerococcus degeneri
Anaerococcus mediterraneensis
Anaerococcus vaginalis
Bacteroides thetaiotaomicron
Corynebacterium striatum
Enterococcus faecalis
Fusobacterium nucleatum
Haemophilus parainfluenzae
Klebsiella oxytoca
Morganella morganii
Others
Parvimonas micra
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Prevotella timonensis
Proteus vulgaris
Staphylococcus aureus
Streptococcus anginosus
Streptococcus oralis
Streptococcus periodonticum
Veillonella parvula

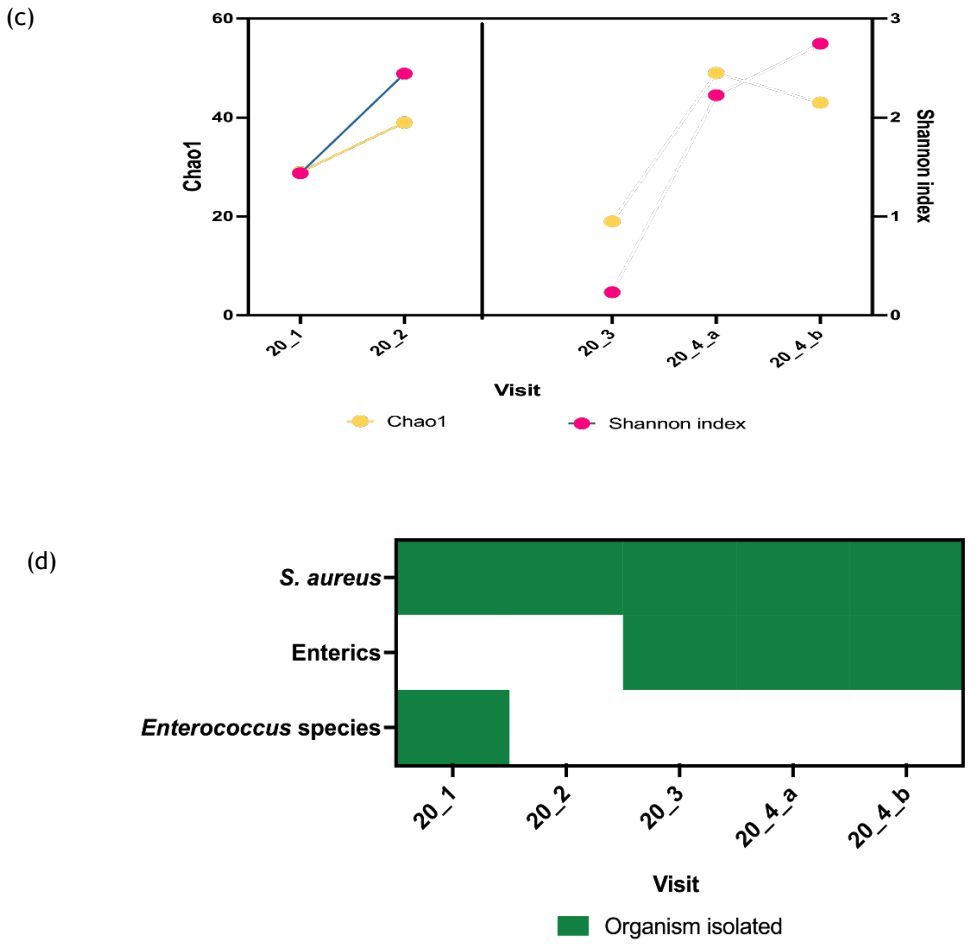


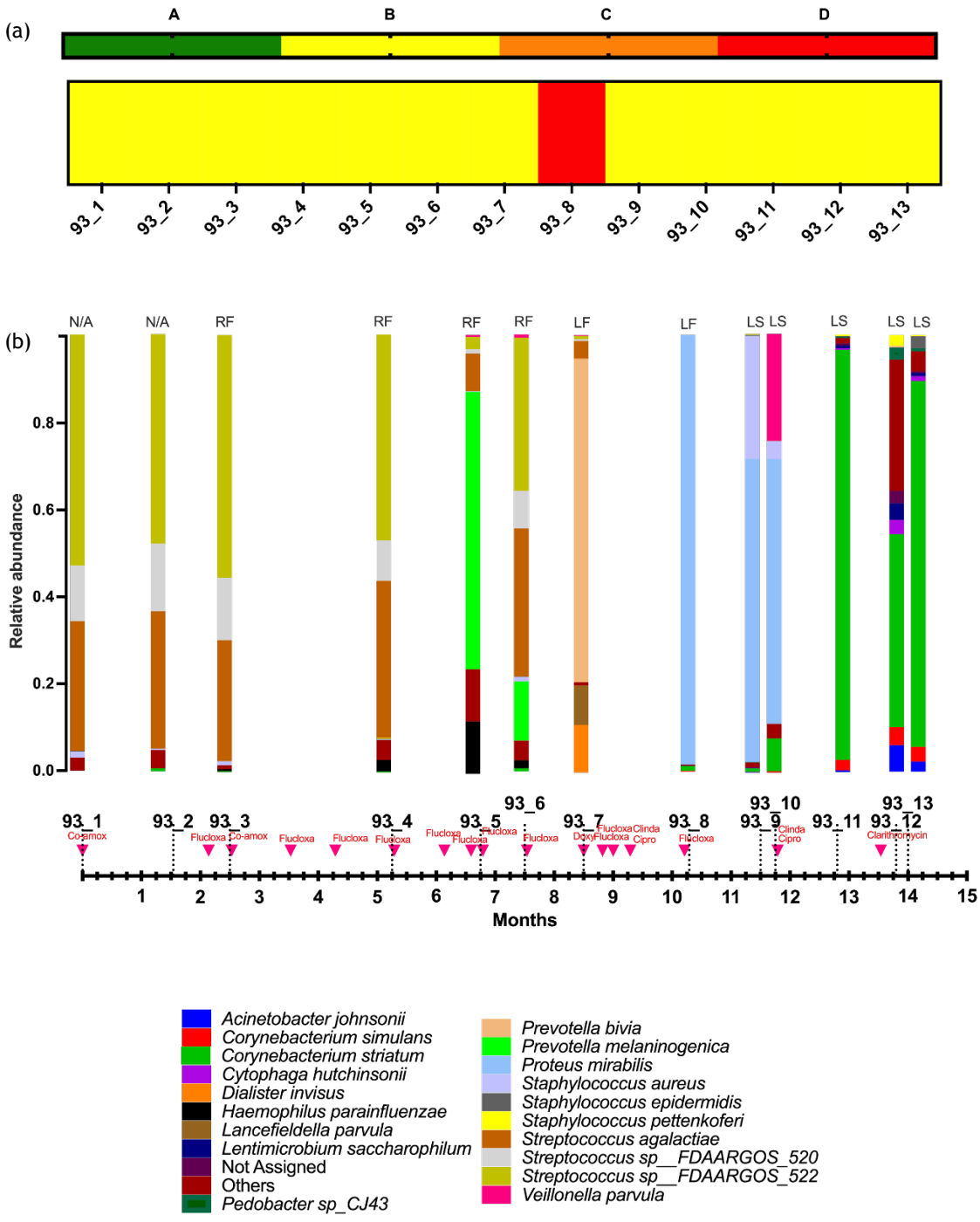
Figure 4.4 Timeline and microbiome profile of patient 20. (a) Stage of infection for each visit. (b) Relative abundance of bacterial species against the visit timeline with antibiotics treatment denoted with pink triangle. Distinct locations of the wound were denoted as left heel (LH), right amputation site (RA) and locations not available (N/A) (c) α-diversity as measured by Chao1 and Shannon index (d) Organisms isolated coloured in green as obtained from standard microbiology culture.

Patient 93

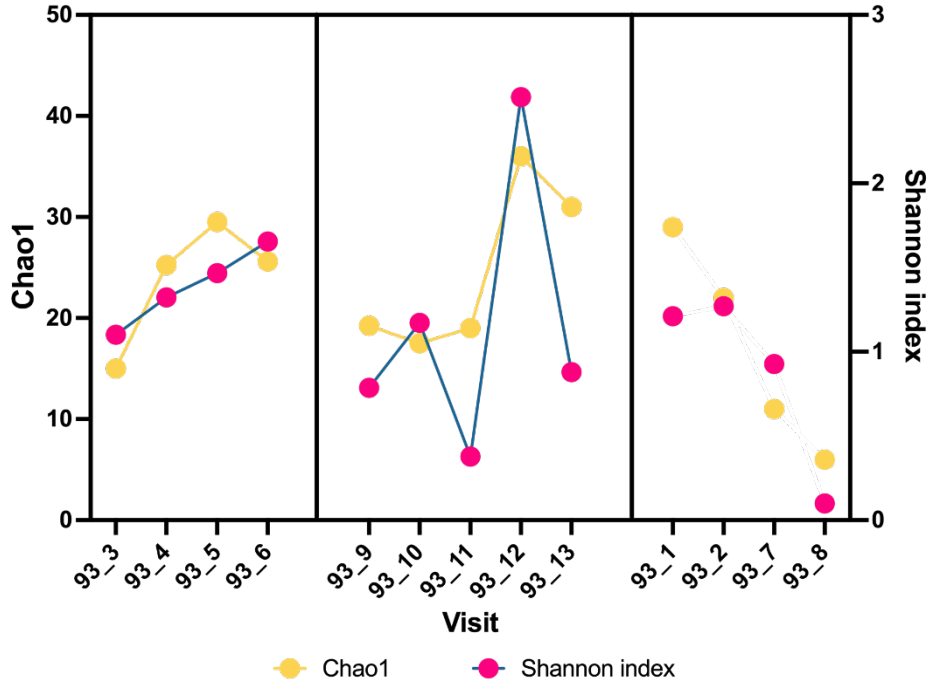
Initially, patient 93 presented with an infected wound that involved 56 weeks of follow-up period (Figure 4.5). During the follow-up visits, multiple wounds were sampled including right first toe, left first toe and left second toe. Since the first two visits did not mention the location of the sampling, appropriate comparisons with subsequent visits were not possible. From visit 3 to 6, the wound involved the right first toe. Microbiome composition from this wound shows a predominant mix species of *Streptococcus*, including *S. agalactiae* during the first two visits. There was an emergence of *P. melaninogenica* that was only detected in visits 5 and 6. *S. aureus* was detected no higher than 1% across all samples. At visit 7, the wound was sampled from the infected left first toe which showed a high abundance of *Prevotella bivia*. This wound underwent a surgical amputation and the follow-up microbiome after the amputation at visit 8 showed a high abundance of *P. mirabilis* (98.3%). The wound was observed to heal in the subsequent visit. However, a new wound occurred adjacent to the healed wound on the second toe. The microbiome of this new wound was dominated by *P. mirabilis* and *S. aureus*. After one week of follow-up, *P. mirabilis* abundance remained the highest while an increased abundance of *Veillonella parvula* and a decreasing abundance of *S. aureus* was observed. Similar to the first toe, the wound also required amputation. Post-amputation microbiome as shown from visit 11 has shown a high increase and persistence of *C. striatum*. The diversity of the right first toe shows an increasing trend as measured by Shannon and Chao1 index, although there was a slight decrease in abundance based on Chao1 on the last visit for the right first toe wound. For the left second toe wound, much higher diversity was observed post-amputation but the opposite was for the left first toe wound.

Similar to the sequencing finding, Group B *Streptococcus* and *S. aureus* were isolated from the right first toe. Both of the organisms have been persistently isolated from 93_3-93_6, except in 93_4 when *S. aureus* showed no growth. For the left first toe, post-amputation (93_8) shows the isolation of mixed skin and coliforms, replacing the growth of *S. aureus* and enterics that were isolated before (93_7). In the left second toe, S. aureus and enterics were persistent in the initial sampling, however, only showed a mixed skin in the repeat cultures (93_11-93_13). As observed in the left first toe, post-amputation for the left second toe also results in the isolation of mixed skin.

The wound was treated repeatedly with Flucloxacillin from visit 2 until 8. Multiple antibiotics including Doxycycline, Clindamycin and Ciprofloxacin were also added to the treatment between visit 7 and 8. At visit 10, Clindamycin and Ciprofloxacin treatment was repeated and switched to Clarithromycin approaching visit 12.



(c)



(d)

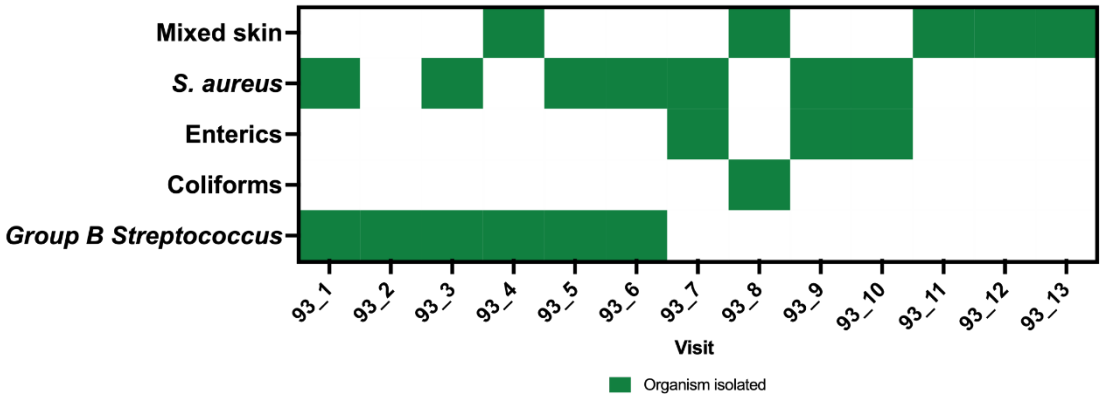
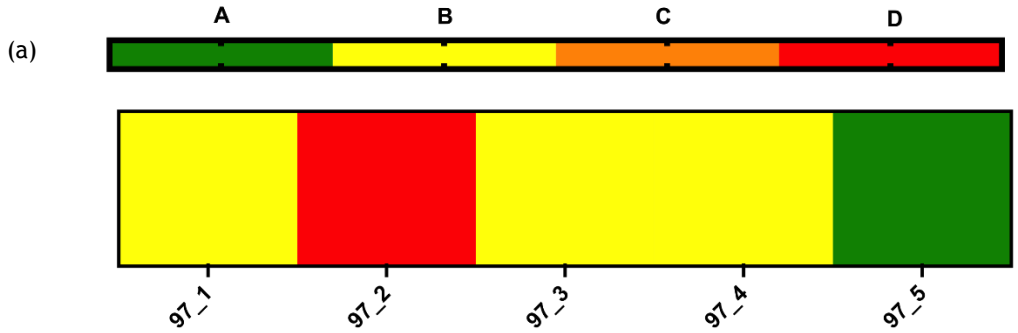
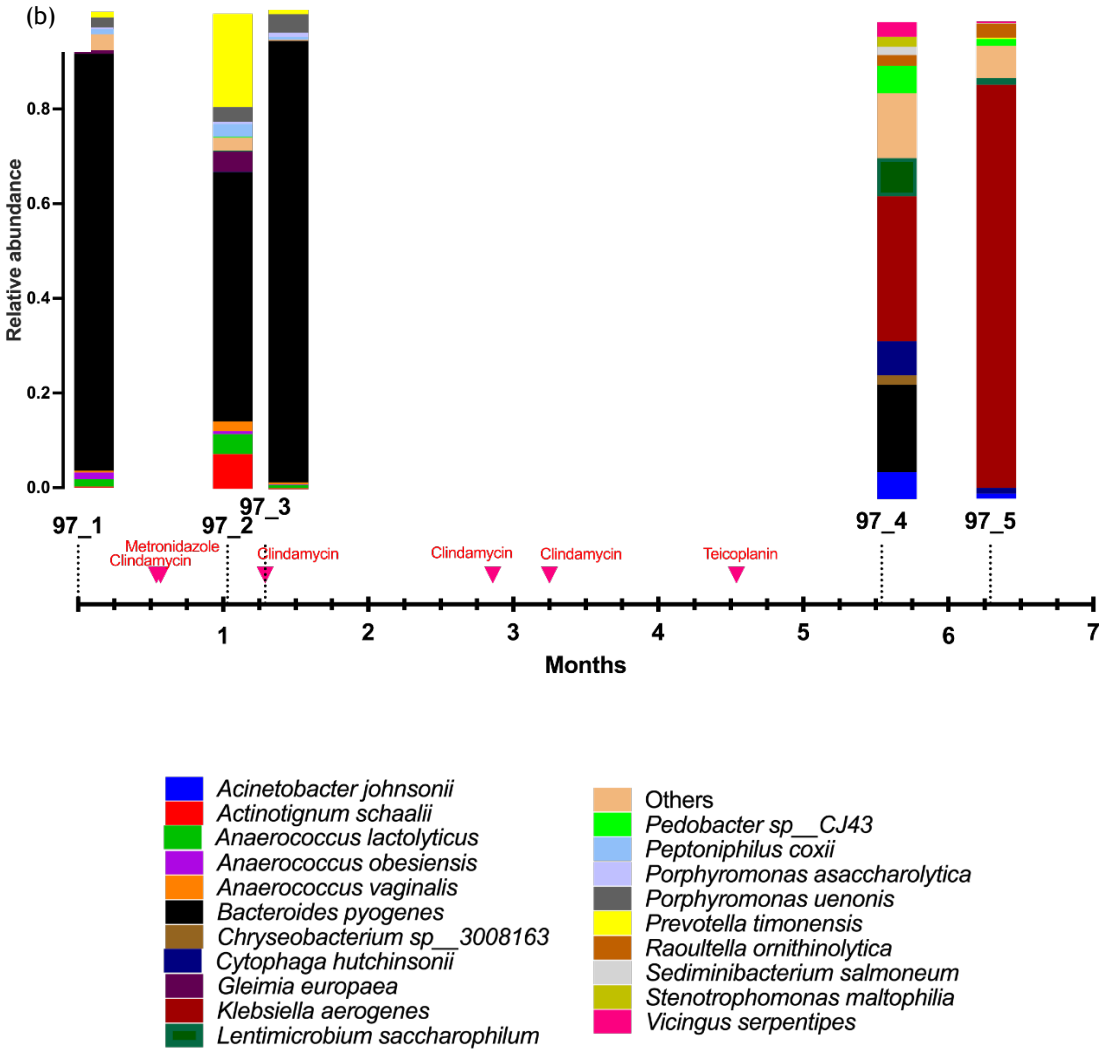


Figure 4.5 Timeline and microbiome profile of patient 93. (a) Stage of infection for each visit. (b) Relative abundance of bacterial species against the visit timeline with antibiotics treatment denoted with pink triangle. Distinct locations of the wounds were denoted as, right first toe (RF), left first toe (LF), left second toe (LS), locations not available (N/A) (c) α-diversity as measured by Chao1 and Shannon index. Samples 93_3 to 93_6 and 93_9 to 93_13 indicate wounds from right foot and left second toe, respectively. (d) Organisms isolated coloured in green as obtained from standard microbiology culture.

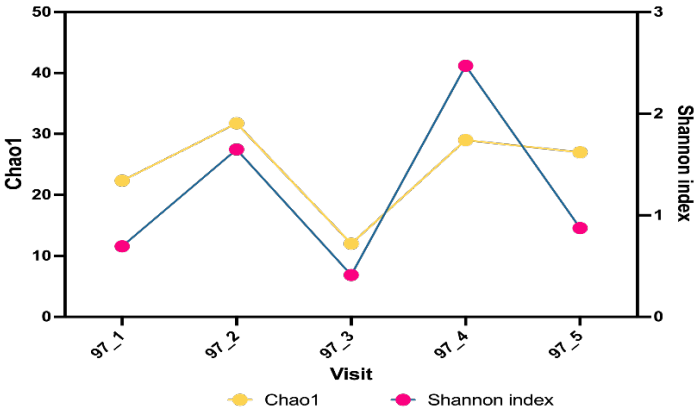
Patient 97

Patient 97 was presented with an infected wound from the big toe and has involved follow-ups before and after amputation. On the first visit, the microbiome was predominated by *B. pyogenes*, an obligate anaerobe, as the most abundant species at 87.6% of relative abundance (Figure 4.6). Metronidazole and Clindamycin were prescribed in between the first and second follow-up. The abundance *of B. pyogenes* decreased to 52.5% in the second visit upon antibiotic treatment, however increased to 92.1% in the third visit. The wound also consisted of other anaerobes including *Prevotella*, *Peptoniphilus* and *Porphorymonas* species. Surgical amputation was performed following visit 3. There was a high reduction of the *Bacteroides pyogenes* to 18.3% following the amputation as collected from the wound in visit 4. The wound showed improvement with undetectable levels of *B. pyogenes* in the last visit. This niche was replaced with an increase in the presence of *Klebsiella aerogenes*. The treatment with anti-anaerobic agents of Clindamycin was prescribed repeatedly until after amputation. The diversity was the lowest in visit 3 when the wound was predominantly consisting of *B. pyogenes*. The average observed taxa before amputation (visits 1, 2 and 3) was lower than post-amputation with a diversity of 22 and 28 as measured by Chao1, respectively. According to standard culture, anaerobes were persistently isolated in the first three visits but were not detected in visit 4 following the amputation. Following amputation, wound was predominated by coliforms.




(b)
Relative abundance
0.0
0.2
0.4
0.6
0.8
97_1
97_2
97_3
97_4
97_5
Metronidazole
Clindamycin
Clindamycin
Clindamycin
Clindamycin
Teicoplanin
1
2
3
4
5
6
7
Months
Acinetobacter johnsonii
Actinotignum schaalii
Anaerococcus lactolyticus
Anaerococcus obesiensis
Anaerococcus vaginalis
Bacteroides pyogenes
Chryseobacterium sp__3008163
Cytophaga hutchinsonii
Gleimia europaea
Klebsiella aerogenes
Lentimicrobium saccharophilum
Others
Pedobacter sp__CJ43
Peptoniphilus coxii
Porphyromonas asaccharolytica
Porphyromonas uenonis
Prevotella timonensis
Raoultella ornithinolytica
Sediminibacterium salmoneum
Stenotrophomonas maltophilia
Vicingus serpentipes

(c)



(d)

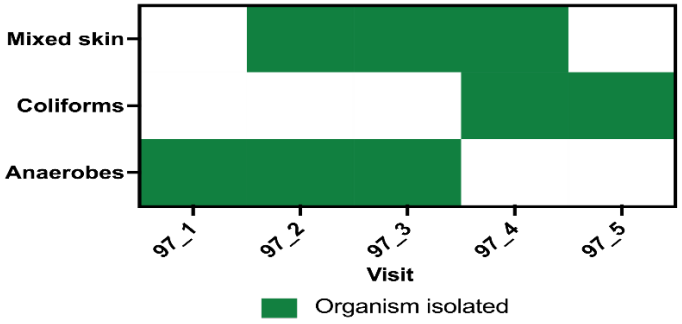
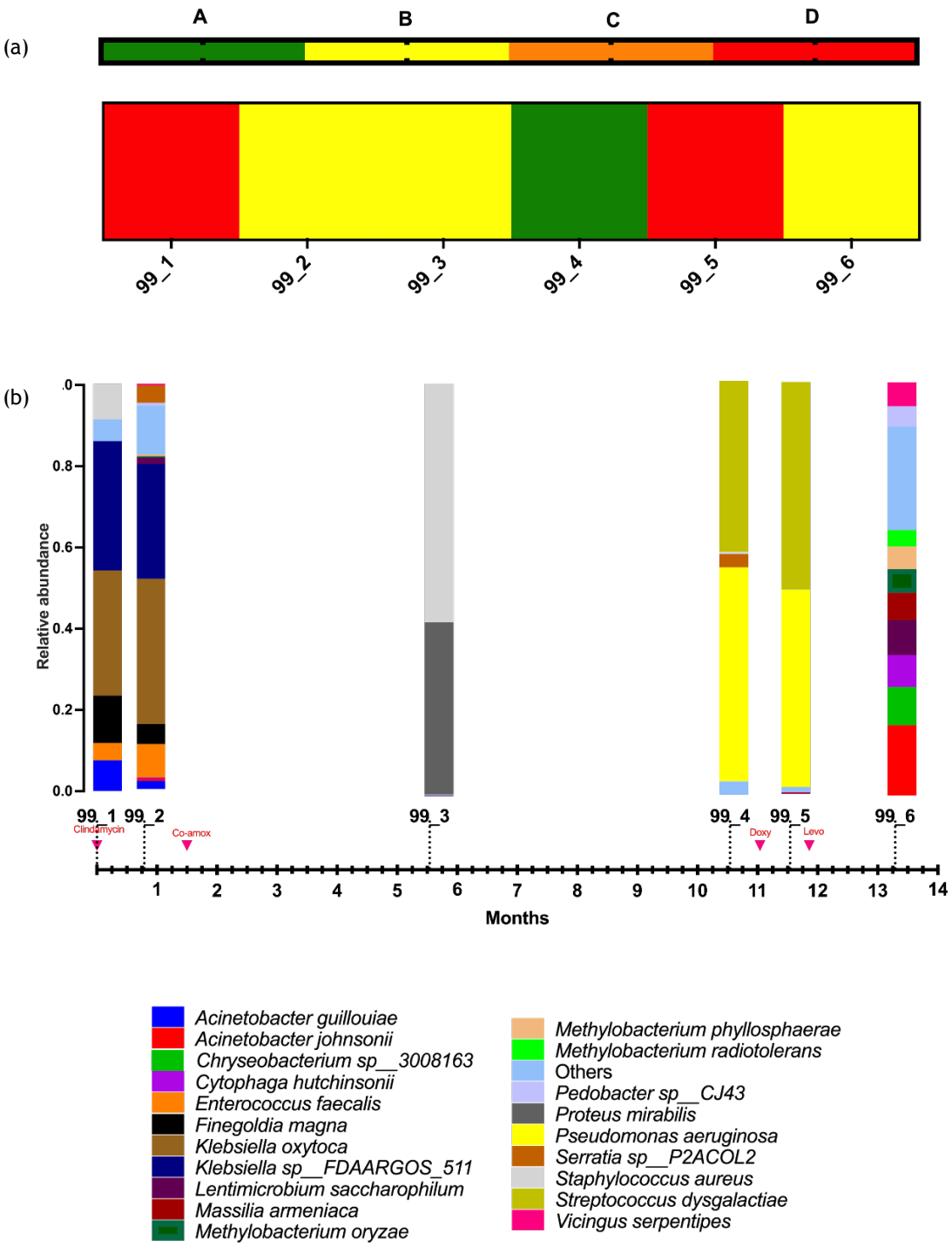


Figure 4.6 Timeline and microbiome profile of patient 97. (a) Stage of infection for each visit. (b) Relative abundance of bacterial species against the visit timeline with antibiotics treatment denoted with pink triangle. (c) α-diversity as measured by Chao1 and Shannon index. (d) Organisms isolated coloured in green as obtained from standard microbiology culture.

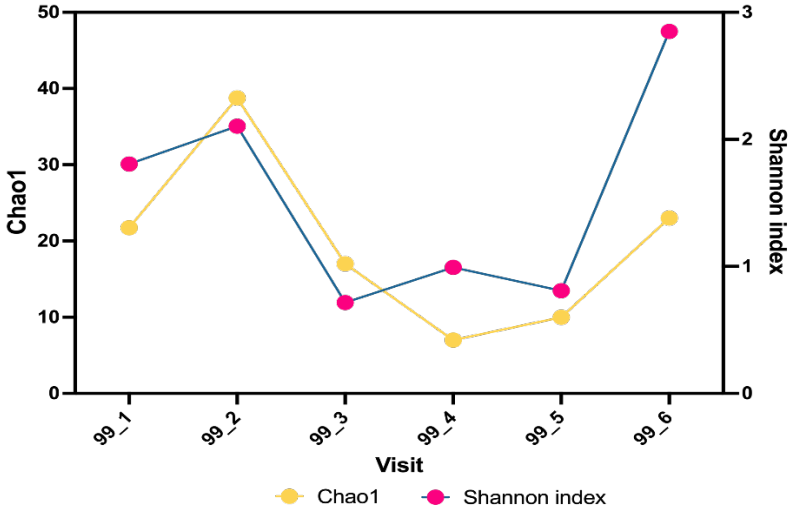
Patient 99

The wound of left big toe was presented from patient 99 with 53 weeks duration of follow-up. The wound microbiomes of the first and the second visit that were taken around ~ 3 weeks apart were almost similar in species composition (Figure 4.7). Both visits show the presence of *Klebsiella* species, *F. magna* and *E. faecalis* among the most abundant species. Apart from that, *S. aureus* was also identified with 8.67% RA in the first visit. The wound was treated with Clindamycin and Co-amoxicillin between the first and the third visit. Subsequent sampling was taken at around 19 weeks after with microbiome predominantly consisted of *S. aureus* (57.8%) and *P. mirabilis* (41.7%). However, the predominant species entirely changed to *S. dysgalactiae* and *P. aeruginosa* in visit 4 and remained the same in the wound taken after 4 weeks (visit 5) despite the treatment with Doxycycline. Following the treatment with Levofloxacin, the microbiome has increased in diversity in visit 6 with the elimination of the previous two dominant species. In contrast, standard culture was reported as no growth from visit 6.

Initially, culture revealed a mixed growth of *S. aureus* and enterics. However, only enterics were isolated in the subsequent culture. The recurrence of *S. aureus* was observed following the initial absence in the previous culture before it was undetectable in the following visit. The isolation of *S. aureus* was intermittent from the culture before reappearing again on the fifth visit until no growth was detected at the last follow-up. Coliforms were also found with *Pseudomonas* species and *Streptococcus* group G in the wound in the two visits. At the final follow-up, no organisms were isolated.


(a)
A
B
C
D
99_1
99_2
99_3
99_4
99_5
99_6
(b)
Relative abundance
0.0
0.2
0.4
0.6
0.8
1.0
99_1
99_2
99_3
99_4
99_5
99_6
Clindamycin
Co-amox
Doxy
Levo
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Months
Acinetobacter guillouiae
Acinetobacter johnsonii
Chryseobacterium sp__3008163
Cytophaga hutchinsonii
Enterococcus faecalis
Finegoldia magna
Klebsiella oxytoca
Klebsiella sp__FDAARGOS_511
Lentimicrobium saccharophilum
Massilia armeniaca
Methylobacterium oryzae
Methylobacterium phyllosphaerae
Methylobacterium radiotolerans
Others
Pedobacter sp__CJ43
Proteus mirabilis
Pseudomonas aeruginosa
Serratia sp__P2ACOL2
Staphylococcus aureus
Streptococcus dysgalactiae
Vicingus serpentipes

(c)



(d)

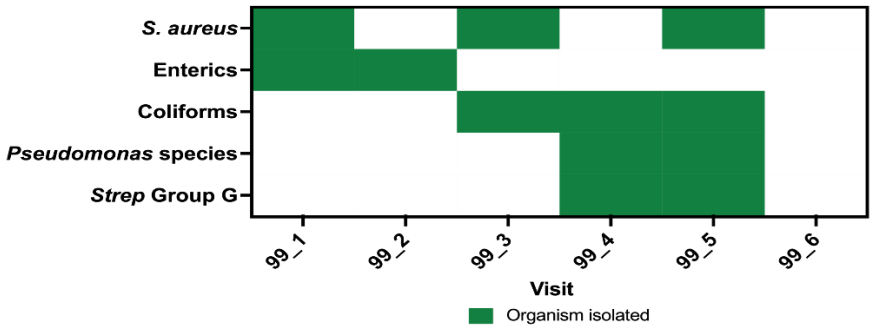
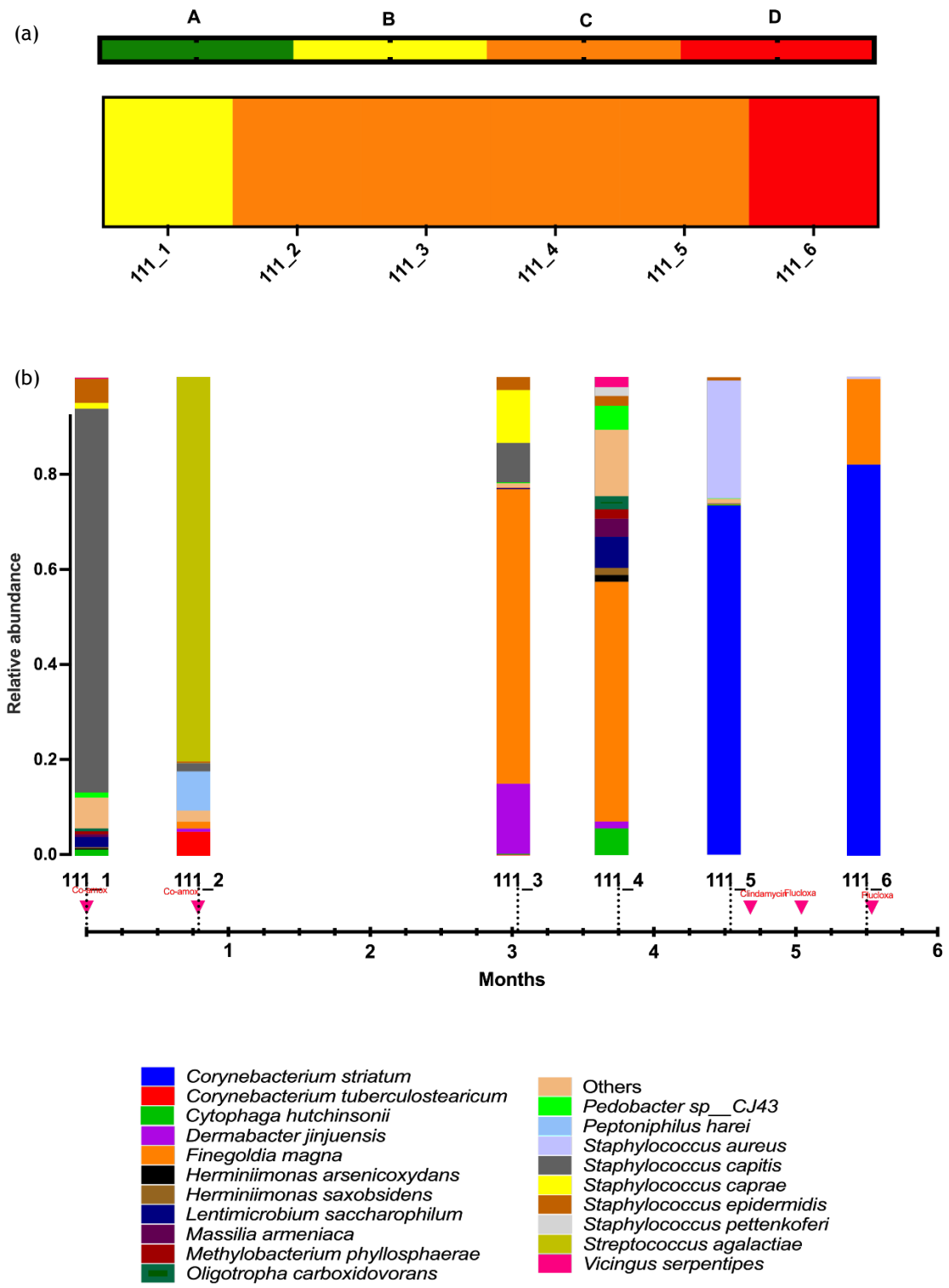


Figure 4.7 Timeline and microbiome profile of patient 99. (a) Stage of infection for each visit. (b) Relative abundance of bacterial species against the visit timeline with antibiotics treatment denoted with pink triangle. (c) α-diversity as measured by Chao1 and Shannon index. (d) Organisms isolated coloured in green as obtained from standard microbiology culture.

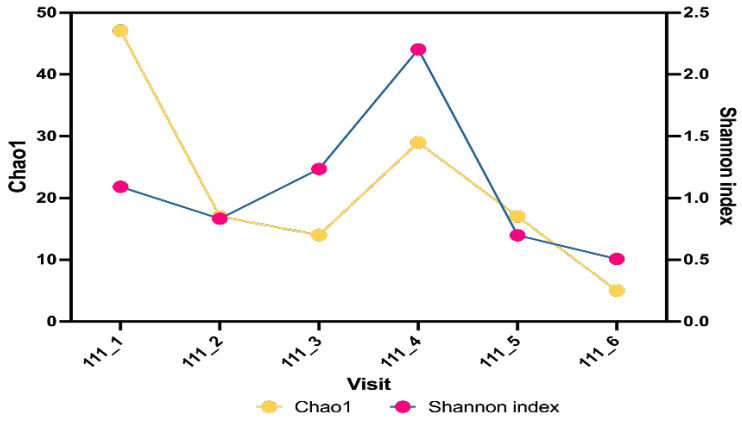
Patient 111

Around 22 weeks of non-healing period, patient 111 underwent six visits regarding an infected wound on their left great toe. The microbiome during the initial visit was the most diverse as measured by Chao1 and Shannon diversity indexes (Figure 4.8). The microbiome was predominated by coagulase-negative *Staphylococcus* (CoNS), with *S. capitis* being the most abundant (80.2%), followed by *S. epidermidis* (5.04%) and *S. caprae* (1.19%). Three weeks later during the second visit, the wound was dominated by *S. agalactiae* (80.3 %) and *S. capitis* decreased to only 1.7%. The patient was prescribed with Co-amoxicillin between first and second visits. There was also the presence of GPAC including *P. harei* and *F. magna* accounted for 8.19% and 1.4%, respectively. Nine weeks after, *F. magna* became the most abundant species at 61.5 % and remained at almost the same RA of 50.1% during the fourth visit until almost undetectable (<1 %) in the fifth visit. After 15 weeks, *F. magna* abundance increased to 17.85 %. The last two visits show the abundance of *C. striatum* at around 70-80%. *S. aureus* was identified during the fifth visit at 24.7% RA but became less than 1% after the treatment with Clindamycin and Flucloxacillin. The within-sample diversity shows a noticeable difference between the first visit and the final visit as the wound progresses to the most severe condition with lower diversity observed in severe wounds.

The first wound culture for the patient only shows the isolation of mixed skin. In the subsequent culture, *Streptococcus* group B was isolated alongside the mixed skin, but was absent in the follow-up cultures. A mixed growth between anaerobes and *Candida* species was observed during visits 3,4, and 6 with the addition of *S. aureus* in the culture findings in visits 5 and 6.


(a)
A
B
C
D
111_1
111_2
111_3
111_4
111_5
111_6
(b)
Relative abundance
0.0
0.2
0.4
0.6
0.8
111_1
111_2
111_3
111_4
111_5
111_6
Co-amox
Co-amox
Clindamycin
Flucloxa
Flucloxa
1
2
3
4
5
6
Months
Corynebacterium striatum
Corynebacterium tuberculostearicum
Cytophaga hutchinsonii
Dermabacter jinjuensis
Finegoldia magna
Herminiimonas arsenicoxydans
Herminiimonas saxobsidens
Lentimicrobium saccharophilum
Massilia armeniaca
Methylobacterium phyllosphaerae
Oligotropha carboxidovorans
Others
Pedobacter sp__CJ43
Peptoniphilus harei
Staphylococcus aureus
Staphylococcus capitis
Staphylococcus caprae
Staphylococcus epidermidis
Staphylococcus pettenkoferi
Streptococcus agalactiae
Vicingus serpentipes

(c)



(d)

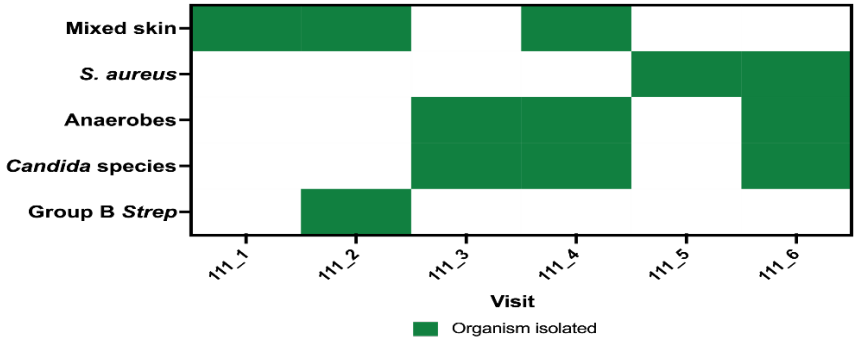
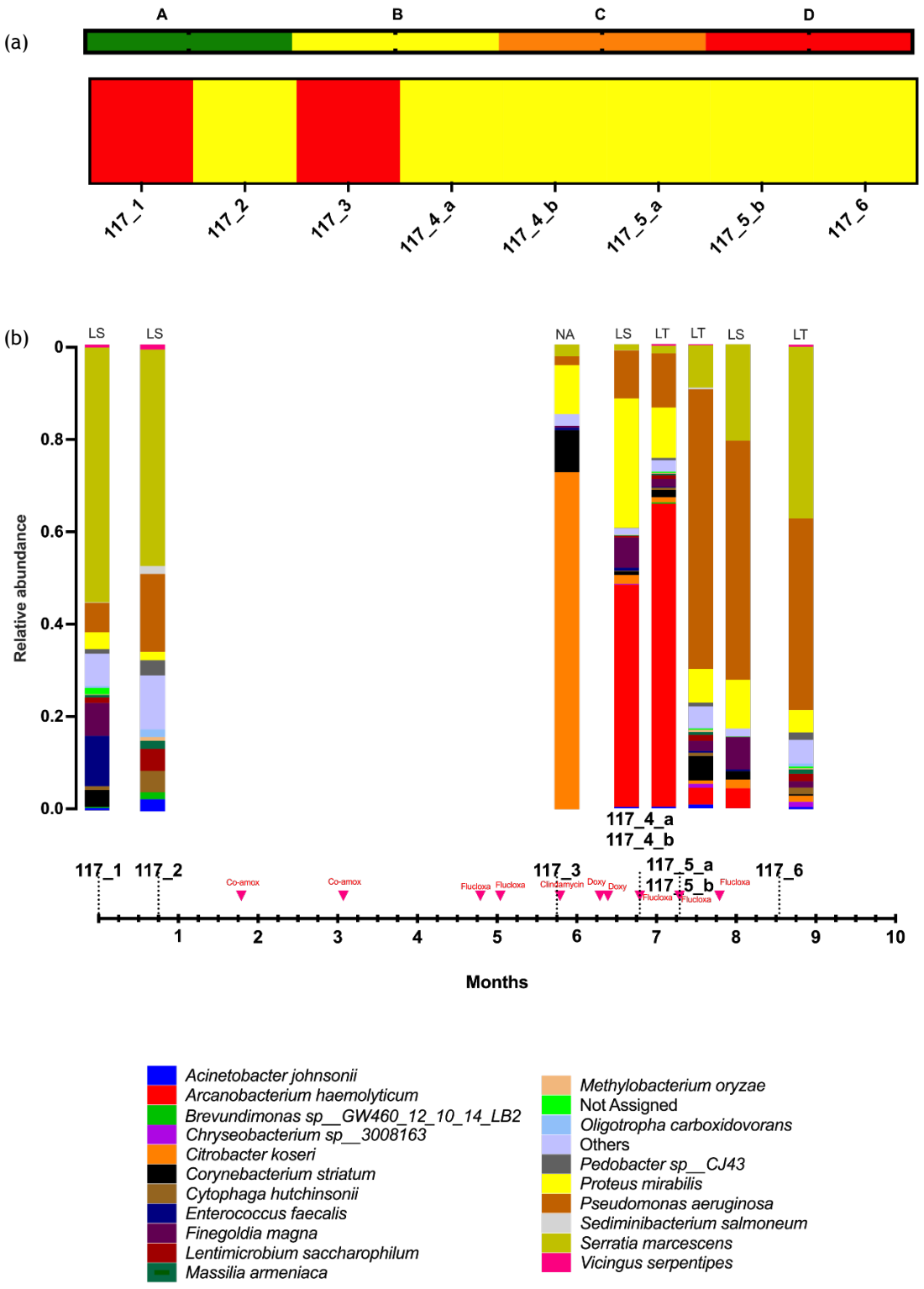


Figure 4.8 Timeline and microbiome profile of patient 111. (a) Stage of infection for each visit. (b) Relative abundance of bacterial species against the visit timeline with antibiotics treatment denoted with pink triangle. (c) α-diversity as measured by Chao1 and Shannon index. (d) Organisms isolated coloured in green as obtained from standard microbiology culture.

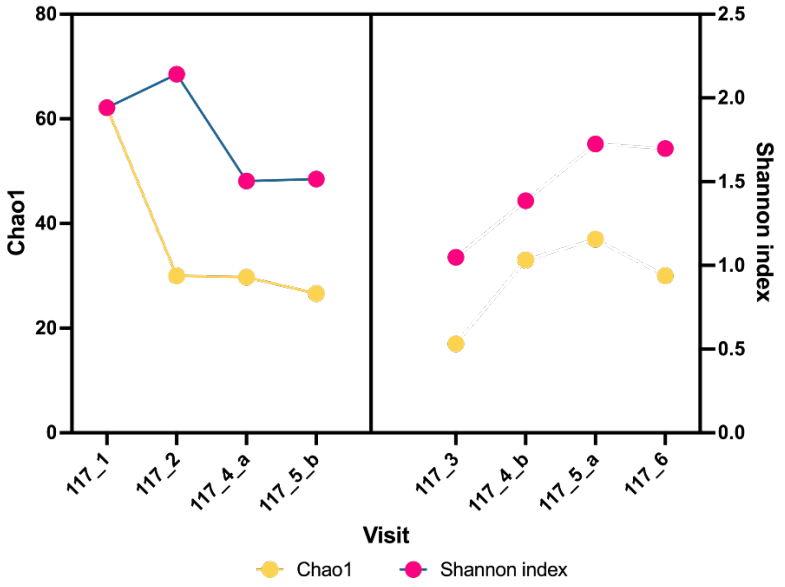
Patient 117

The wound microbiomes described in Figure 4.9 represent the wounds collected from the adjacent wound of either the second or third toe of the left foot except for the microbiome of the third visit that was collected from the right foot. The wound microbiomes in this patient appeared to have conserved species that appeared in every visit. These species include *P. aeruginosa*, *S. marcescens* and *P. mirabilis*. When looking at the standard culture, a similar group of bacteria was observed. The relative abundance of these species fluctuates between visits but was always present despite the treatment with antibiotics including Co-amoxicillin, Flucloxacillin, Clindamycin and Doxycycline. After weeks from the first presentation, *A. haemolyticum* was identified in the wound and accounted for the most abundant species for both the second and left-toed wounds. Despite remaining persistent in the wounds, the abundance of *A. haemolyticum* decreased during the subsequent visit with an increasing abundance of *P. aeruginosa* following the treatment with Flucloxacillin. The diversity of the wound from the left toe shows a decreasing trend as measured by the Chao1 index. Flucloxacillin was repeatedly prescribed for the patient from visit 3-6.

When observing the standard culture, the left second toe initially revealed the presence of coliforms. In the follow-up culture, *Pseudomonas* species were observed with coliforms. Repeated cultures showed the continued presence of *Pseudomonas* species. Similarly, in the left third toe, *Pseudomonas* growth was observed alongside the enterics. The wound has remained infected throughout the study period.


(a)
A
B
C
D
117_1
117_2
117_3
117_4_a
117_4_b
117_5_a
117_5_b
117_6
(b)
LS
LS
NA
LS
LT
LT
LS
LT
Relative abundance
0
0.2
0.4
0.6
0.8
117_4_a
117_4_b
117_1
117_2
Co-amox
Co-amox
Flucloxa
Flucloxa
117_3
Clindamycin
Doxy
Doxy
117_5_a
117_5_b
Flucloxa
Flucloxa
Flucloxa
117_6
1
2
3
4
5
6
7
8
9
10
Months
Acinetobacter johnsonii
Arcanobacterium haemolyticum
Brevundimonas sp__GW460_12_10_14_LB2
Chryseobacterium sp__3008163
Citrobacter koseri
Corynebacterium striatum
Cytophaga hutchinsonii
Enterococcus faecalis
Finegoldia magna
Lentimicrobium saccharophilum
Massilia armeniaca
Methylobacterium oryzae
Not Assigned
Oligotropha carboxidovorans
Others
Pedobacter sp__CJ43
Proteus mirabilis
Pseudomonas aeruginosa
Sediminibacterium salmoneum
Serratia marcescens
Vicingus serpentipes

(c)



(d)

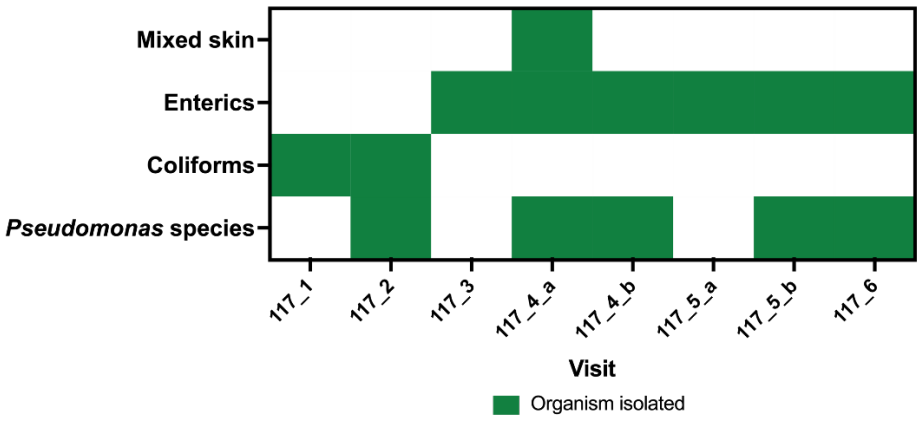
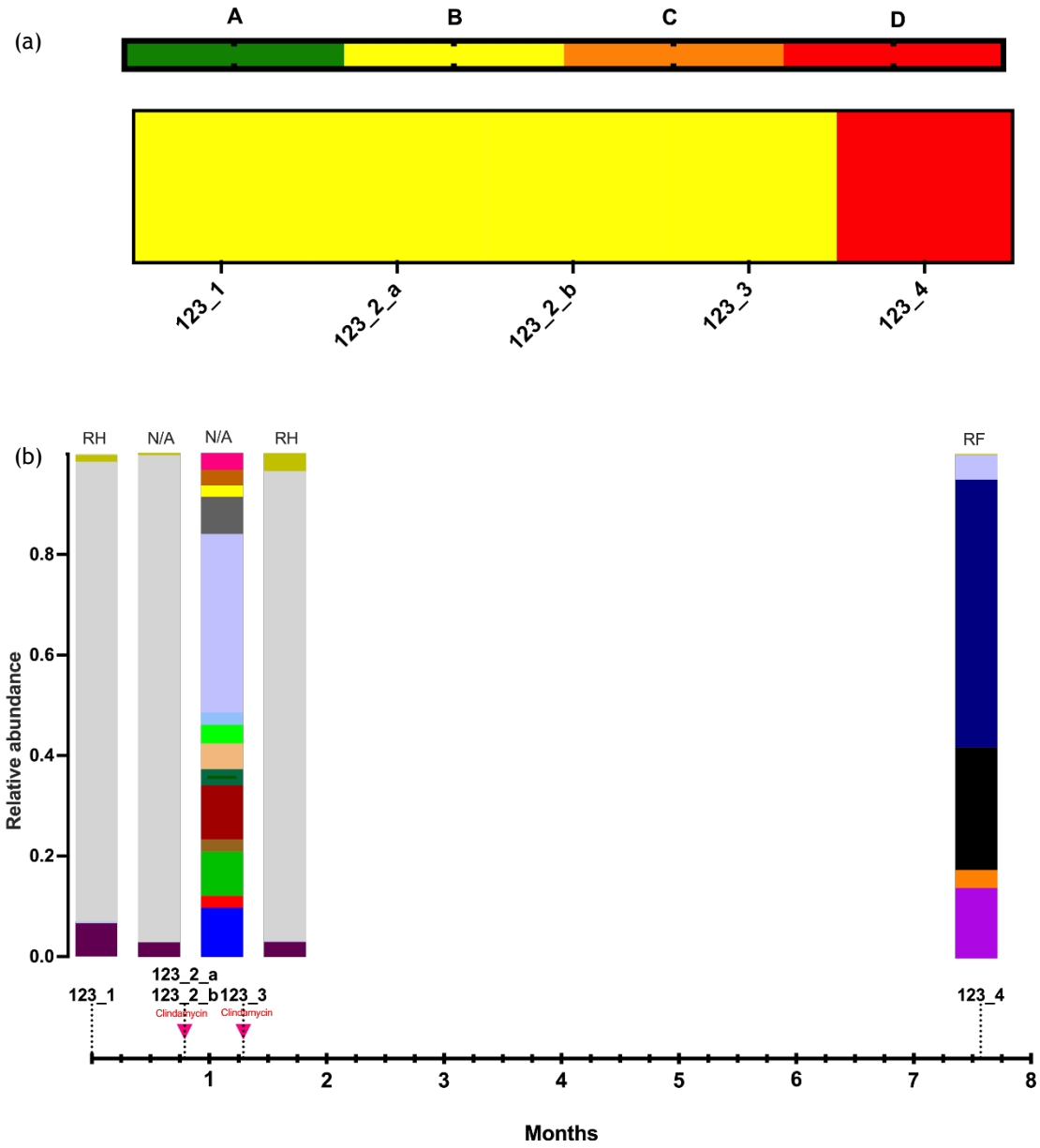


Figure 4.9 Timeline and microbiome profile of patient 117. (a) Stage of infection for each visit. (b) Relative abundance of bacterial species against the visit timeline with antibiotics treatment denoted with pink triangle. Distinct locations of the wound were denoted as left second toe (LS), left third toe (LT), location not available (N/A) (c) α-diversity as measured by Chao1 and Shannon index. (d) Organisms isolated coloured in green as obtained from standard microbiology culture.

Patient 123

Initially, an infected wound was presented on the right heel, which was predominated by CoNS (*S. capitis* and S. *epidermidis*) and *Kocuria koreensis* (Figure 4.10). In less than a month, two locations of the wounds were sampled; the right second toe and right heel. Microbiomes between these two locations were compositionally different. Unfortunately, due to missing data on the sampling location, it was not possible to distinguish the microbiome of these two locations. However, one of the locations has a very similar composition as the first visit while the second sample was more diverse with infrequent species potentially from the environment including *A. johnsonii*, *L. saccharophilum* and *C. hutchinsonii*. After two weeks, only the right heel sample was collected and showed the same dominant taxa as the first visit. There was a decline in the diversity from the first and the third visit as measured by Chao1 (49 to 4) and Shannon index (0.35 to 0.30). The wound was treated with Clindamycin prior to visit 3 follow-up. The wound remained infected during all the visits. The final wound from this patient was collected from the forefoot after 23 weeks. At this stage, the wound has become ischemic and involves the bone. The predominant taxa identified were *K. pneumoniae*, *F. magna* and *Enteroccocus faecalis* in descending order of relative abundance. Organisms isolated from the standard culture show mostly mixed skin for the first three visits with no organism isolated in one of the samples from visit 2. *Candida* species were isolated alongside enterics from the wound sample at the last visit. The only antibiotic treatment that was prescribed for the patient was Clindamycin between the second and the third visit.


(a)
A
B
C
D
123_1
123_2_a
123_2_b
123_3
123_4
(b)
RH
N/A
N/A
RH
RF
Relative abundance
0.0
0.2
0.4
0.6
0.8
123_1
123_2_a
123_2_b
123_3
123_4
Clindamycin
Clindamycin
1
2
3
4
5
6
7
8
Months
Acinetobacter johnsonii
Brevundimonas sp__DS20
Cytophaga hutchinsonii
Enterococcus faecalis
Enterococcus gilvus
Finegoldia magna
Gaiella occulta
Klebsiella pneumoniae
Kocuria koreensis
Lentimicrobium saccharophilum
Massilia armeniaca
Methylobacterium oryzae
Methylobacterium phyllosphaerae
Oligotropha carboxidovorans
Others
Pedobacter sp__CJ43
Pedomicrobium americanum
Phyllobacterium zundukense
Staphylococcus capitis
Staphylococcus epidermidis
Vicingus serpentipes

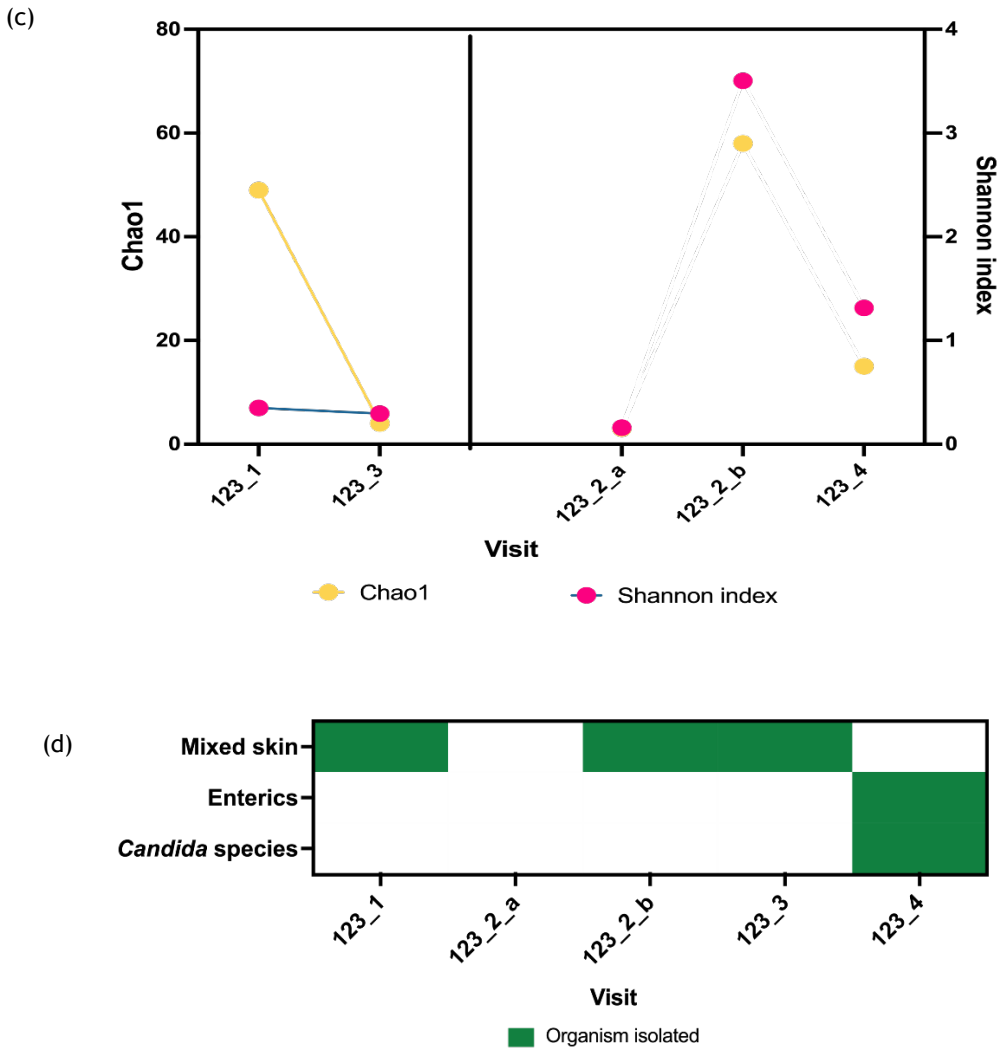


Figure 4.10 Timeline and microbiome profile of patient 123. (a) Stage of infection for each visit. (b) Relative abundance of bacterial species against the visit timeline with antibiotics treatment denoted with pink triangle. Distinct locations of the wound were denoted as, right heel (RH), right forefoot (RF), location not available (N/A) (c) α-diversity as measured by Chao1 and Shannon index. (d) Organisms isolated coloured in green as obtained from standard microbiology culture.

4.4.3 Diversity changes post-amputation

Surgical amputation of the infected wound has an impact on the diversity of the wound microbiome. Figure 4.11 compares the α-diversity as measured by Chao1 and Shannon diversity index from samples collected a visit before the amputation was performed and during a follow-up visit after the procedure. There was no similar pattern between the wounds before and after the amputation. The wound from the right toe of patient 10 showed a slight decrease as measured by Chao1(22 to 19) but increased in diversity post-amputation based on the Shannon index (1.33 to 1.76). Patient 93 has undergone two events of surgical amputation from the first and second toe of the left foot. The diversity of both wounds has decreased post-amputation when measured by the Shannon index with the first and second toe showing the index of 0.93 to 0.1 and 1.17 to 0.38, respectively. However, the wound from the left second toe shows a slight increase as measured by the Chao1 index, from 17.5 to 19. On the other hand, the wound from patient 97 shows an increase in diversity post-amputation with both diversity indexes. Because the follow-up visit was not controlled for all wounds, the diversity measured post-amputation will represent wound samples that were collected at different times. The sample collection time was 4,7,10 and 17 weeks for left second toe (Patient 93), left first toe (Patient 93), right toe (patient 10) and great toe (Patient 97), respectively.

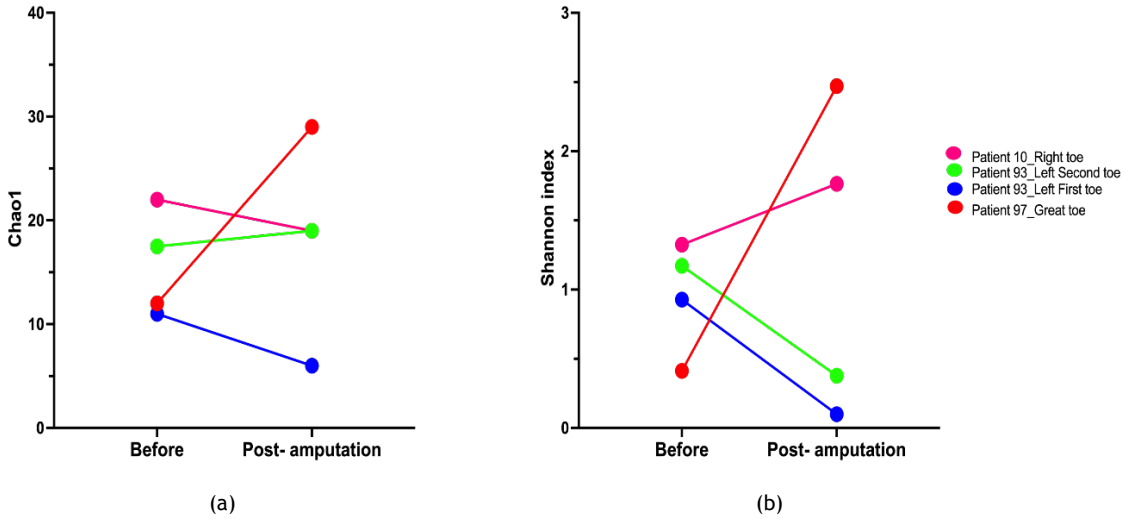


Figure 4.11 Diversity measured before and post-amputation. The before and post-amputation diversities were measured according to (a) Chao1 and (b) Shannon index. Four wound locations that were amputated were indicated by the respective colours.

4.5 Discussion

In reviewing the literature, few studies have focused on the dynamics of DFU microbiomes. Based on the data from Chapter 3, DFU undeniably consists of vast species of bacteria of various origins. In fact, the open nature of the lesion would continuously be exposed to opportunistic bacteria at any time, both from the skin (endogenous) and wider environment (exogenous). Many of the studies have characterised the microbiomes from a single time point, which is counter intuitive considering the chronicity of DFU and cross-sectioning the microbiomes at any time point may have omitted important information about community structure of the wound as time progresses. Several factors that influence the microbiome have been explored in many studies, and the use of antibiotics is one of them (Langdon et al., 2016). In this chapter, the microbiome is shown to be patient specific, and fluctuation of species composition and transitioning between species can occur over time, which may relate to antibiotic treatment and patient specific factors.

Chronicity of the wound was evident in this present study based on the long follow-up period of the wounds, that can continue for several months to over a year in some patients. The wound duration in this study does not represent the entire episode of the infection, but is rather limited by the study period. DFU of an average of 6 months and up to 84 months ulcer duration has been reported elsewhere (Musa & Ahmed, 2012). By following the wounds' progress across multiple visits, there was an apparent pattern of stability in the bacterial community at some points between the visits for most patients. The period of stable microbiomes can persist at any time during the wound duration (Loesche, 2016). These stable microbiomes show the predominance of at least two dominant species that last for a few visits of ~3 weeks and up to over a year. It seems possible that a stable microbiome is due to the established (mature) formation of biofilm that is often tolerant to treatment in the wound. Moreover, animal studies have shown that biofilm can be formed as early as 3 hours by *S. aureus* and 8 hours by *P. aeruginosa* (Percival et al., 2015). These observations corroborate the hypothesis of Dowd and colleagues (2008) who suggested that the chronicity of the wound was driven by a pathogenic biofilm produced by a group of bacteria that symbiotically live together rather than an action of a single species (Dowd, Wolcott, et al., 2008). Furthermore, these results reflect those of Loesche and

colleagues (2017) who also found that community-type of wound microbiome can persist for multiple visits with an average transition between community-type was longer in unhealed or wound that require amputation (~4 – 6 weeks) compared to healed wounds (~2 weeks) (Loesche et al., 2017). It is difficult to explain the persistence microbiomes that appeared for certain duration despite the antibiotic treatment, but it might be related to tolerance to treatment, a phenomenon commonly seen in biofilms (Pouget et al., 2020). It has been shown that sessile minimum inhibitory concentration can increase up to 100 times the planktonic MIC (Tuon et al., 2023) and this concentration usually cannot be achieved in tissue (Crowther et al., 2021). According to these data, we can infer that a persistent mixture of bacteria species implies for an aggressive treatment to remove the biofilms, though antibiotics alone may not be sufficient. Indeed, active debridement is essential to support chemotherapeutic approaches.

All patients were treated with various antibiotics as listed in Table 4.1. It was not surprising to see Flucloxacillin, Co- amoxicillin and Clindamycin had been used more often in patients than any other antibiotics like Linezolid, Metronidazole and Teicoplanin. Most of the empirical antibiotics are primarily targeting the most common pathogen associated with DFU mainly gram-positive cocci including *S. aureus* and *Streptococcus* species (Senneville et al., 2024). Antibiotics such as Metronidazole are primarily used to treat anaerobic infections (Löfmark et al., 2010), such as in a wound from patient 97 that consisted of *B. pyogenes*. Despite antibiotic treatment, some infected wounds required amputation. In wounds that healed after amputation, the microbiome shows a reduction of anaerobes which was thought to be the key species that drive the infection in patient 97. However, this current study is unable to determine the endpoint or 'healthy' microbiome of the individual patients due to the unavailability of a comparator from an unaffected site. Previous studies have shown a less diverse bacterial community in wounds compared to non-wounded skin on the contralateral side(Gardiner et al., 2017). However, wounds involved in the study were clinically non-infected and the patients had no recent exposure to antibiotics (Gardiner et al., 2017). Despite being limited by healthy controls, this current study was able to observe a shift in diversity as the wound progresses. However, the diversity between visits does not appear to have a fixed trend, as some wounds can increase or decrease in diversity at any time point. It is possible that a robust trend cannot be seen due

to the coverage and impact of the antibiotics treatment that affecting on the specific bacteria in the wound. It is in fact in line with a study by Price and colleagues (2009) who found that the impact of antibiotics may not entirely change the whole composition of the microbiome, but rather affect certain species to a certain extent (Price et al., 2009). As a result, diversity will change when microbial composition is affected. The impact of antibiotics on the changes in the microbiome has been similarly reported in healed and non-healed wounds (MacDonald et al., 2019). Despite the prolonged use of antibiotics, the wounds remain chronic in this study. The benefit of systematic antibiotics in routine use for wound healing is still unclear (O'Meara et al., 2000), though there are clear implications for antimicrobial stewardship and antimicrobial resistance.

It is important to note that patient 2 was treated with Fluconazole for the infected wound. This patient has had a very consistent microbiome throughout 74 weeks. Following a week of antifungal treatment, the wound remained clinically infected with no major changes in dominant species. In contrast to a study of 17 patients with wounds with suspected fungal infections, the majority of the cases were healed or improved following antifungal treatment without any concomitant use of antibiotics (Heald et al., 2001). It has also been reported in the same study that relapse of infection has been observed when antifungal treatment was stopped. Although *Candida* species were also isolated from wounds of patients 10,18, 111 and 123, no antifungals were prescribed. The implication of antifungal treatment in this current study is very limited to be able to observe any further benefit, though based on the data in the earlier chapter the implications of fungi in wounds is greater than perceived. It may also be of interest for future studies to investigate the combination of antibiotic and antifungal treatment in cases where fungi are present. This may not only improve treatment outcomes but also prevent biofilm formation at the early stage.

Amputation is necessary to stop the spreading of infection to the healthy tissue. In patients 10 and 97, both cases show a high abundance of anaerobes in the wound prior to amputation including *P. bennonis*, *P. harei* and *F. magna* in patient 10 while patient 97 consisted of *B. pyogenes* as the most abundant anaerobe apart from *Prevotella* and *Porphyromonas* species. Similarly, the wound from the left first toe of patient 93 also shows a high abundance of anaerobe mainly *P. bivia*. The high abundance of anaerobes in wounds that require amputation has similarly

been reported elsewhere with *Bacteroides* was significantly associated with amputation (Mudrik-Zohar et al., 2022). In the left second toe of patient 93, *P. mirabilis* was relatively higher than *V. parvulla*, an obligate anaerobe that has rarely been reported from diabetic foot (N. Singh & Yu, 1992). Interestingly, the presence of closely related genera of *Proteeae* and *V. parvulla* in the same wound is similar to the previously reported case of foot ulcer that co-isolates *P. morganii,* now known as *M. morganii* (Borchardt et al., 1977). Those who have undergone amputation have been found to have 3% more *Proteus* in their wound (Dörr et al., 2021). In the same study, the relative risk for amputation is increased to 20% when anaerobes are present. Nevertheless, the pathogenic role of anaerobes remains unclear although the detection of anaerobes in infected wounds has been reported from various studies in the last decade (Charles et al., 2015). Following amputation, there was a notable reduction in anaerobes and an increase in enteric bacteria. Despite being prevalent in the GI tract, enterics have been associated with common skin colonisers below the waist as they were in proximity to the anorectal area (Ki & Rotstein, 2008). Therefore, the observation of predominantly enteric bacteria in the DFU might suggest the restoration of the microbiome. Similarly, in patient 93, post-amputation resulted in a high abundance of *C. striatum* which is also commonly found as a skin coloniser (Martínez-Martínez et al., 1997).

Wound diversity before and after amputation does not show a conclusive pattern in this study. As previous studies have shown a more diverse population in intact skin compared to the wound (Gontcharova et al., 2010), it was hypothesised that amputation would result in a more diverse population. However, not all wounds in this study increased in diversity following amputation. These differences could be explained by the time of collection for the sample after the amputation. Increasing diversity was observed in samples collected at 10 and 17 weeks, but not during 4 and 7 weeks based on the Shannon index alone. This means, the later the wound was sampled, the more diverse it would be. In a study following sharp debridement, a significant reduction in diversity, as measured by the Shannon index, was observed in wounds collected at two weeks after the procedure in wounds that healed by 12 weeks (L. R. Kalan et al., 2019). Despite amputation, healing wounds have been suggested as remission because new ulcers can recur in 40% of cases in one year, 60% within three years, and 65% within 5 years period

(Armstrong et al., 2017; Khan et al., 2020). In this study wounds from patients 2, 20 and 123 are recurrent ulcers following previous amputation. Unfortunately, a comparison between the microbiome of old and recurrent ulcers was not possible in this study as previous wounds occurred before the start of this study.

DFUs can appear as single or multiple ulcers at various locations of the foot including the tip of the toe, plantar, dorsum and digital with multiple occurrences of ulcers associated with poor prognostic outcomes (Ndosi et al., 2018). Similarly, some patients in this study have multiple wounds on the same foot and the opposite foot. Another clinically relevant finding in this study was wound microbiomes are unique to each foot. However, there will be instances where certain species will present on both feet suggesting some overlap. The current data highlight the importance of sampling every infected wound so as not to overlook the species that are colonising each site. As a result, the finding would potentially consolidate wound treatment management. Furthermore, the overlapping of bacteria across wounds was more pronounced in adjacent sites on the same foot. This is possible due to the proximity of the location that made the dissemination of the bacteria feasible. It is difficult to compare the microbiomes of different locations that were taken on different visits as they may be influenced by the antibiotics used during the period. Therefore, observation was based on wounds that were sampled together during the same visit. It does provide some insights into consideration of intra-patient infection control procedures.

To date, studies have focused on the dynamic of bacterial composition with the influences of clinical interventions such as antibiotic treatment and surgical amputation. As shown in patients 2, 10, 18, 111 and 123, some wounds were also colonised by *Candida* species. Due to the limitation of the 16S primers, *Candida* species was unable to be compared relative to the bacterial composition. Unlike bacteria, the presence of *Candida* species was sporadic in all patients. These results are in agreement with findings by Kalan and colleagues (2016) which showed the fluctuation of the fungi taxa in patients across multiple visits. Although transient colonisation may be one of the reasons, synergistic interaction between *Candida* and bacteria is also possible (L. Kalan et al., 2016).

It is impossible to sterilise the DFU from the colonising microbes as an open wound is constantly exposed to microbial contamination. Moreover, the aim of managing

the DFU with antibiotic therapy is only indicated when the wound is clinically infected (Senneville et al., 2024). This reflects the important roles of skin microbiota in wound healing at maintaining healthy skin barrier (Yang et al., 2024). From all the swabs collected, wound microbiomes were constantly composed of various bacteria species. In fact, even several weeks following amputation, the predominant species still prevailed in the wound as observed in patient 97. Therefore, vigilant wound care assessment is important in distinguishing exacerbation of infection or progression of the healing process.

Clearly, DFU microbiomes consist of a dynamic consortium of bacteria that are unique and specific to individual patients. Longitudinal analysis of the microbiome from multiple visits and multiple wounds has highlighted the importance of clinical assessment for each wound over time as resilience to antibiotic treatment may prolong the healing process which could lead to poor clinical outcomes. Intra-patient dissemination of pathogenic microbiota is likely between wounds, thus early recognition of newly formed ulcers and the prevention of cross-contamination could prevent another episode of chronic wound. Furthermore, wounds would always be colonised by microbes, therefore the stability of the microbiome with severed clinical manifestation might be indicative for an aggressive treatment intervention. Further research that defines the pathogenicity role of the microbiome and interaction with the host would help in improving the understanding of the complex dynamic in chronic wound microbiome.

Chapter highlights:

Each wound microbiome is unique and patient specific.

Antibiotic treatments caused little change in bacteria composition.

The presence of fungi should not be ignored when managing chronic wounds.

5 Characterising Fungi in Diabetic foot ulcer

5.1 Introduction

Diabetic foot ulcer (DFU) has a worrying outcome among diabetic patients with amputation was reported to be 1 in 400 (Kerr et al., 2019). In addition, the financial burden of managing the ulcer and amputation was estimated to cost up to £962 million in the UK (Kerr et al., 2019). Bacteria are mostly identified as the main pathogens in cases of infected DFU while fungi remain understudied. That being said, numerous skin microbiota comprised of bacteria and fungi have been found on the skin of both diabetic and non-diabetic individuals (Redel et al., 2013) which makes the lack of fungal wound studies a puzzling omission in the field. From a bacterial perspective, skin commensals exist and provide a protective barrier from invading pathogens. For example, *S. lugdunensis* produces antimicrobial molecules such as lugdunin that will inhibit the growth of *S. aureus* (Byrd et al., 2018). On the other hand, an amicable interaction between *Propionibacterium* with *S. aureus* may lead to biofilm formation (Byrd et al., 2018). In many reported cases, *S. aureus* has been repeatedly isolated from the wounds (Sadeghpour Heravi et al., 2019) with capacity to form biofilms accounted as one of its key virulence attributes (Mamdoh et al., 2023). However, instead of rendering a singular species for the infection, Malone *et al.*, show that a chronicity of the wound has been implicated by the presence of polymicrobial biofilms (Malone, Bjarnsholt, et al., 2017) and this often demonstrated a high tolerance to antimicrobial treatments. Fungi have been demonstrated in chronic wounds apart from bacteria (L. Kalan et al., 2016).

The generalisation that DFU wound healing is halted because of only bacterial pathogens should be re-examined closely as vast species of fungal species were reported to cause infection as summarised in Chapter 1. These studies were mostly based on microbiology culture with some limited studies using 18S or ITS regions to scrutinize fungi from infected wounds. Moreover, the emergence of multi-drug resistant fungi such as *Candida auris* has led to a renewed awareness of the threat of *Candida* species as a critical fungal pathogen (World Health Organization, 2022). Such microorganisms are able to form biofilms (Sherry et al., 2017) and persevere for a long duration in the environment (Welsh et al., 2017). Furthermore, many *in vitro* models of multi-species biofilms in chronic wounds were still limited to only bacteria species with only *C. albicans* included in one model of 11 species biofilms (J. L. Brown et al., 2022; Short et al., 2023). Other

simpler wound models include triadic species of *S. aureus*, *P. aeruginosa* and *C. albicans* (E. M. Townsend et al., 2016). Studies have shown that multi-species biofilms involving fungi were more tolerant compared to mono-species counterparts (Kean et al., 2017; Ramstedt & Burmølle, 2022). Thus, this previous work justifies the necessity to account for fungi when managing chronic DFU and potentially leading to a more realistic and representative wound model.

A complete picture of the microbial landscape in the wound can only be perceived by collating data for both bacterial and fungal species present. However, as touched upon above, the DFU mycobiome is understudied leaving the fungal community to remain speculative in depicting its clinical significance. Hence, recognising their presence in parallel with bacterial populations is vital in delineating an accurate and precise microbial community which underpins the chronicity of the wound. Therefore, this chapter will focus on the isolation of fungi from DFU wounds using enhanced mycology culture.

5.2 Aims

This chapter aims to detect fungi in diabetic foot ulcers using enhanced mycology culture and qPCR alongside standard microbiology culture. Drawing upon the polymicrobial existence of fungi and bacteria in wounds from the previous chapter, this chapter will explore the relationship by analysing the coexistence between them in culture. Finally, the fungal isolates will be characterised phenotypically for their capacity to form biofilms and susceptibility against conventional antifungals to compare the tolerance profiles between planktonic and sessile forms.

Data in this chapter has been submitted and is under review for publication in:

Allkja J, **Ahmed Bakri**, Short B, Gilmour A, Brown J, Bal AM, Newby K JM, Jenkins T, Short RD, Williams C and Ramage G. 2024. Investigating the prevalence of fungi in diabetic ulcers: an under-recognised contributor to polymicrobial biofilms. APMIS.

Additionally, data from this chapter has been presented at the following conferences:

Ahmed Bakri, Abdullah Baz, Short B, Allkja J, Brown J, Smith P, Williams C, Ramage G. 'Hidden in plain sight: Fungi on diabetic foot ulcer and implication for treatment'. 57th British Society for Medical Mycology (BSMM), Manchester, United Kingdom, September 2023.

Ahmed Bakri, Short B, Butcher M, Allkja J, Delaney C, Brown J, Williams C, Ramage G. 'Characterising fungi from diabetic foot ulcer'. 11th Trends in Medical Mycology (TIMM), Athens, Greece, October 2023.

5.3 Materials and methods

5.3.1 Study participants

A total of 127 patients with diabetic foot ulcers from the Royal Lancaster Infirmary (RLI) were recruited in this study resulting in 349 total number of wound samples. Data involving age and sex were collected as presented in Chapter 2.

5.3.2 Clinical sample collection and processing

Swabs were collected by the attending podiatrist during the clinic appointment. Assessment of the location and condition of the wound was recorded based on the University of Texas wound classification system as presented in Chapter 2. Briefly, wounds were categorised according to the grades 0, I, II and III which correspond to the wound depth and also the stages A, B, C and D that indicate the presence of infection or ischemia. For standard culture as part of standard wound care for the patients, the wound swabs were processed by the biomedical scientists at the microbiology department of RLI. Two additional swabs were collected in a dry empty vial and 50% glycerol separately. The swabs were identified based on the patient number labelled on the sample tube alongside the collection date. These swabs were transported on ice to Glasgow Dental Hospital and School for further processing. An accompanying list of samples was also shipped together during the sample transportation for verification of the sample identifier. All swabs were kept at -80℃ upon arrival until processed.

5.3.3 DNA extraction

Dry wound swabs were thawed at room temperature before being processed. DNA was extracted from the swabs using MasterPure™ Yeast DNA purification kit (LGC Biosearch Technologies, UK) according to the manufacturer's protocol with some modifications. Initially, the wound swabs were immersed in yeast lysis solution and sonicated for 15 minutes prior to the heating step at 65℃ for another 15 minutes. Once completed, the lysate was drawn out from the swabs by gently squeezed against microfuge tube to maximize the volume of the sample collected before discarding. Following that, samples were placed on ice for five minutes and were mixed with MPC protein solution by vortexing. Cellular debris was pelleted at >10000 rpm for 10 minutes and liquid supernatant was transferred to

a new microfuge tube. Isopropanol was added with gentle mixing and then centrifuged at >10000 rpm for 10 minutes leaving the DNA pellet at the bottom of the tube. The supernatant was removed and replaced with 70 % ethanol to wash the pelleted DNA. The 70% ethanol was prepared in nuclease-free water (Life technologies, UK). Next, ethanol was removed by pipetting and then with centrifugation at >10000 rpm for 10 seconds to remove and dry the DNA pellet. Finally, TE buffer was added to suspend the DNA. All the reagents used for the extraction were supplied in the kit except for the isopropanol and ethanol.

5.3.4 Quantification of fungal load by qPCR

The extracted DNA was then quantified using internal transcribed spacer (ITS) primers with the following sequences; ITS3 Uni F (5'-TCGCATCGATGAAGAACGCAGC - 3') and ITS4 Uni R (5' – TCTTTTCCTCCGCTTATTGATATGC – 3') in which the sequences can also be found here https://www.glasgowbiofilms.co.uk/. Using a StepOnePlus™ qPCR machine (Life Technologies, UK), each reaction containing 1 µL of DNA and 19 µL of master mix that consist of 10 µL 2x qPCRBIO SyGreen Mix Hi-ROX (PCR Biosystems Ltd., London, UK) , 7 µL of Invitrogen™ Ultrapure™ DNase/RNase-Free Distilled water (Life Technologies, UK) and 1 µL each of forward and reverse primers at 10 µM were carried out with the following thermal profiles for 40 cycles: Initial denaturation at 50 ℃ for 2 minutes, annealing at 95 ℃ for 2 minutes followed by cycling stage which involves 95 ℃ of denaturation at 2 minutes then annealing at 50 ℃ for 30 seconds. The cycle threshold value (Ct) was used to determine the colony forming equivalence (CFE) per millilitre by comparing to the standard curve generated from *C. albicans* SC5314 with a PCR efficiency of >90%.

5.3.5 Standard microbiology culture

As part of standard care, wound swabs were processed for microbiology culture according to the standard microbiology protocol at the Microbiology Department of RLI. Data from standard microbiology culture were collected by Dr Ana Casey from RLI.

5.3.6 Enhanced mycology culture

The DFU swabs maintained in glycerol at -80 ℃ were thawed at room temperature and vortex mixed before culture. 50 µL of the samples were inoculated and spread out on SAB supplemented with chloramphenicol and CHROMagar™ Malassezia (CHROMagar, France). Inoculated plates were then incubated at 30℃ for 14 days with every alternate day observation for fungal growth until the media plates were discarded. Fungal isolates were purified on SAB agar for identification. Yeast-like isolates were identified using MALDI-ToF for accurate identification at the Microbiology Department of Glasgow Royal Infirmary. All fungal isolates were stored at -80 ℃ in cryobeads.

5.3.7 Biofilms quantification by crystal violet

To assess the biofilms formed by the fungal isolates, a crystal violet assay was carried out to measure the biomass of the biofilms. Fungal isolates were grown in YPD medium (Sigma-Aldrich, UK) for 18 to 24 hours at 30℃ in a shaker incubator. The cells were centrifuged at 3500 rpm for five minutes before discarding the supernatant and were washed with sterile PBS followed by another five minutes of centrifugation. The supernatant was then removed, and the cells were suspended in 10 mL of sterile PBS (Sigma-Aldrich, UK). Fungal cells were counted with a haemocytometer followed by standardising to 1 x 10^6 cells in Roswell Park Memorial Institute (RPMI-1640) medium (Sigma-Aldrich, UK). Subsequently, 200 µL of the inoculum was dispensed into the 96-well flat bottom plate and incubated at 37 ℃ for 24 hours. After the incubation, biofilms were washed with PBS and allowed to air dry overnight at room temperature. A 0.05% crystal violet was used to stain the biofilms formed at the bottom of the well for 20 minutes followed by a gentle washing step with tap water to remove the excess stain. 100 µL of absolute ethanol was then added to the well for de-staining before 75 µL was transferred to a fresh 96-well microtiter plate to measure the absorbance at a wavelength of 570 nm with a plate reader.

5.3.8 Planktonic and sessile MIC

Three conventional antifungals including Fluconazole (Sigma- Aldrich, Gillingham, UK), Caspofungin (Sigma- Aldrich, UK) and Amphotericin B (Sigma- Aldrich, UK) were dissolved in either water or DMSO to a stock concentration according to the

manufacturer's instruction. All the antifungals were serially diluted in RPMI medium to give a concentration range from 128 – 0.25 µg/mL before adding the fungal inoculum for planktonic MIC. 100 µL of serially diluted antifungals were dispensed into a 96-well round bottom microtiter plate. Fungal inoculum was standardised to 2 x 10^4 cells in RPMI before being added to the prepared antifungal plate. The final concentration of antifungals tested ranged from 64 – 0.0125 µg/mL. Following a 24-hour incubation at 37℃, the MIC was visually examined as the lowest concentration with no visible growth. When tested with Fluconazole, a trailing effect was observed for some isolates and was analysed with a spectrophotometer at 530 nm for 50% inhibition instead of visual reading.

For assessing the sessile MIC, biofilms were grown as described in 5.3.7 and the antifungal effect was measured as described previously (Ramage et al., 2001). After the biofilms were washed, serially diluted antifungals ranging from 128 – 0.25 µg/ml were added to the well containing biofilms. RPMI alone was added as a growth control and as a negative control. After 24-h incubation at 37℃, the antifungals were removed and were replaced with XTT (2,3-bis(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide; ThermoFisher Scientific, UK) with 1 µM menadione for 2 hours incubation at 37℃ in the dark. Following the incubation, 75 µL of the supernatant was transferred to a fresh 96-well flat-bottom microtiter plate and was read using spectrophotometer at a wavelength of 492 nm to determine the 50 and 90% of biofilm inhibition. The treated biofilms were removed from excess XTT reagent and allowed to dry overnight before assessing the antifungal effect on biofilm biomass using crystal violet assay as described above.

5.3.9 Statistical analysis

Graphs and statistical analysis were performed using Graphpad Prism (Version 8.4.3; GraphPad Software Inc., La Jolla, CA). Student t-test was used to compare the mean difference between two groups. One-way ANOVA was used to analyse the differences in fungal load across grades and stages. A p-value of <0.05 was considered statistically significant.

5.4 Results

5.4.1 Patient demographics

A total of 127 patients were enrolled in this study. Table 5.1 shows the basic demographic of the patients that were involved in this study. Majority of the patients were male (78%) compared to female (22%). The median age of the total patients irrespective of gender was 67 years old.

Table 5.1 Patient demographics of the patient cohort. Various characteristics were documented from the 127 patients included in the study. The age and sex of the patients were included, including the median (interquartile range [IQR]) or % of population for each characteristic.

Characteristics	N = 127
Age	**67 (56, 75)**
Sex	
Male	**99 (78%)**
Female	**28 (22%)**
Median (IQR), n(%)	

5.4.2 Severity of the wounds

All the wounds were classified according to the University of Texas classification system. Figure 5.1 shows the number of samples corresponding to the grades and stages of the wound involved in this study. A total of 349 samples were assessed in this study. However, 10 and 13 samples were unavailable to process for enhanced and standard culture respectively. The wound grade represents the extent of tissue that was affected including the absence of skin break (Grade 0) to the severe one that penetrates the bone (Grade III). The wounds collected were mostly superficial, grade I (57%) followed by grade III (25.8%), grade II (16.3%) and grade 0 with less than 1%. Infected wounds, stage B (54.7%) were predominant in

this study compared to healthy wounds, Stage A (31.2%). The most common combination of grade and stage is BI (infected superficial wound) with 26.6% followed by uninfected superficial wounds AI with 25.5%.

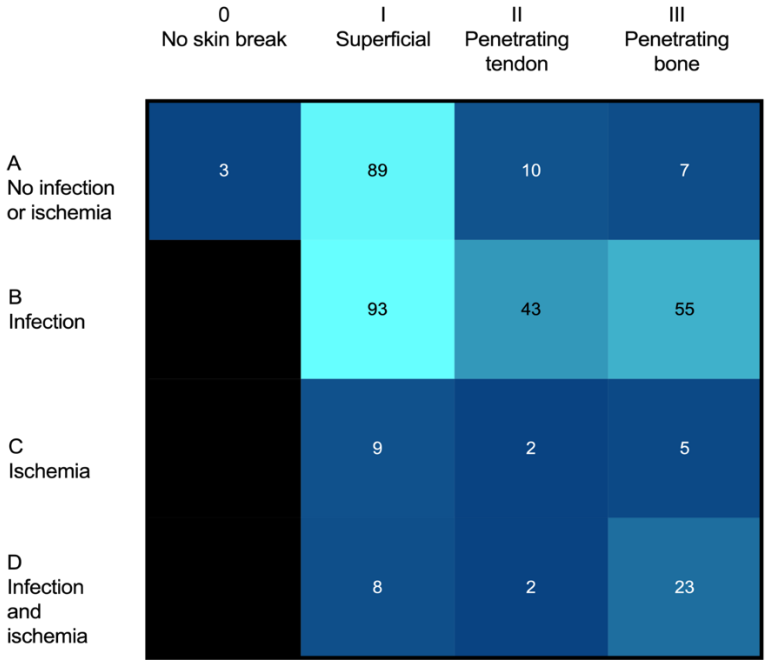


Figure 5.1 Severity of the wounds. A total of 349 wound swabs were classified according to the University of Texas wound classification system.

5.4.3 Fungal burden

Next, the fungal burden was assessed using qPCR. There was a significant difference ($P < 0.05$) of fungal positivity between ITS and culture method. Figure 5.2 shows the percentage of detection from each method. Standard and enhanced culture showed no significant difference in fungal detection. Although there were no significant differences between the culture methods, there were discordant results between them. Eight fungal positive samples were detected in standard culture but not in enhanced culture.

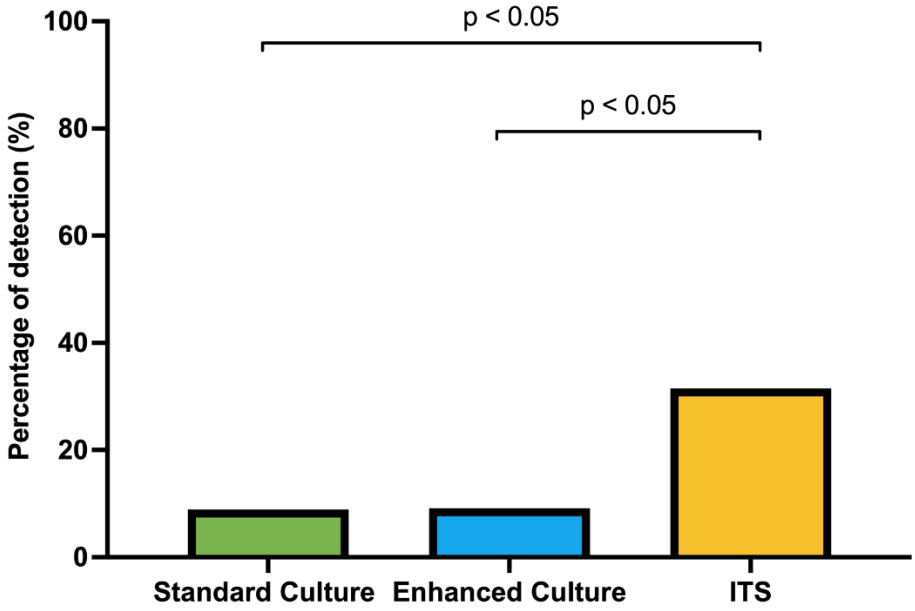


Figure 5.2 Fungal positivity detected from culture and qPCR. Percentage of fungal detection based on standard culture (8.93% ; 30 out of 336), enhanced culture (9.14% ; 31 out of 339) and ITS (31.52% ; 110 out of 349 samples). ITS shows statistically significant difference (P < 0.05) when compared to both standard and enhanced culture by using Fisher exact test.

Fungal burdens were divided into low, intermediate and high loads based on the quartile range of the CFE/mL. Low fungal load was considered as less than Q1 (87.74 CFE/mL) and high if it was more than Q3 (1083 CFE/mL). Range in between Q1 and Q3 was considered as intermediate. The majority of the enhanced culture positive fell into intermediate to high fungal load (Figure 5.3 (i)). The fungal ITS tends to be higher than the culture method for most of the samples Figure 5.3 (ii). When compared between stage and grades (Figure 5.4), fungal burden measured by qPCR were similar.

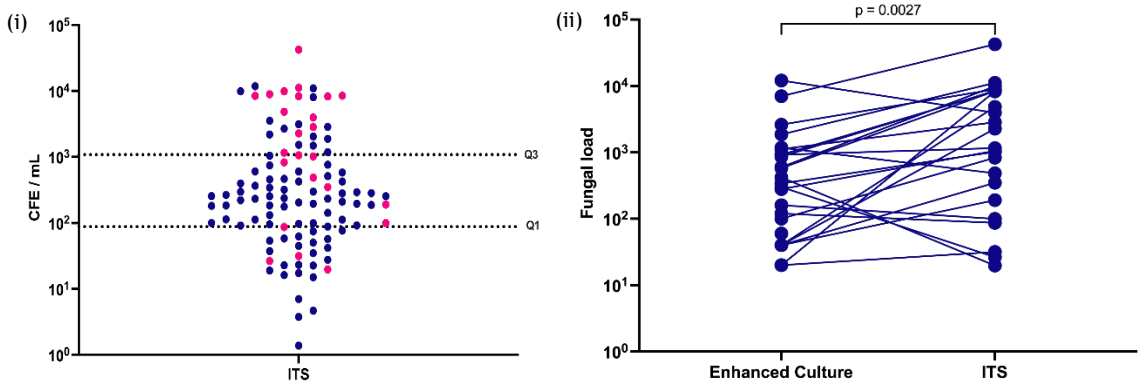


Figure 5.3 Fungal load. A total of 110 wound swabs were detected with fungal DNA. The red point represents the growth of fungi from enhanced culture. Seven samples from enhanced culture were not represented here due to undetected fungal DNA (i). There is a significant difference (p = 0.0027) of fungal load between culture and ITS (ii).

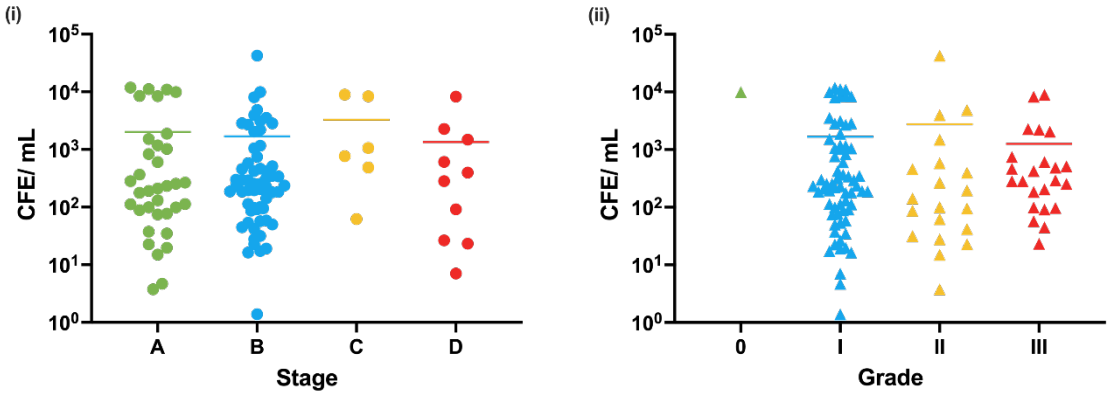


Figure 5.4 Similar fungal burden across the grades and stages. Fungal burdens were measured using ITS 3 and ITS 4 primers using qPCR. The results were determined using a standard curve of *C. albicans* SC5314 and expressed in CFE/ mL. There were no statistical significance differences ($p > 0.05$) observed between the fungal burden of different stages and grades as performed by One-way ANOVA.

All the fungal isolates identified from standard and enhanced culture were yeast-like organisms primarily *Candida* species with only one sample grew *Rhodotorula mucilaginosa*. *C. parapsilosis* complex accounted for 64.5% of the total isolates followed by 22.6% of *C. albicans*. *C. glabrata* represents 9.7% of the culture positive. *C. parapsilosis* was found in all grades and stages (Figure 5.5). Despite the use of CHROMagar™ *Malassezia*, none of the samples were isolated with *Malassezia* species.

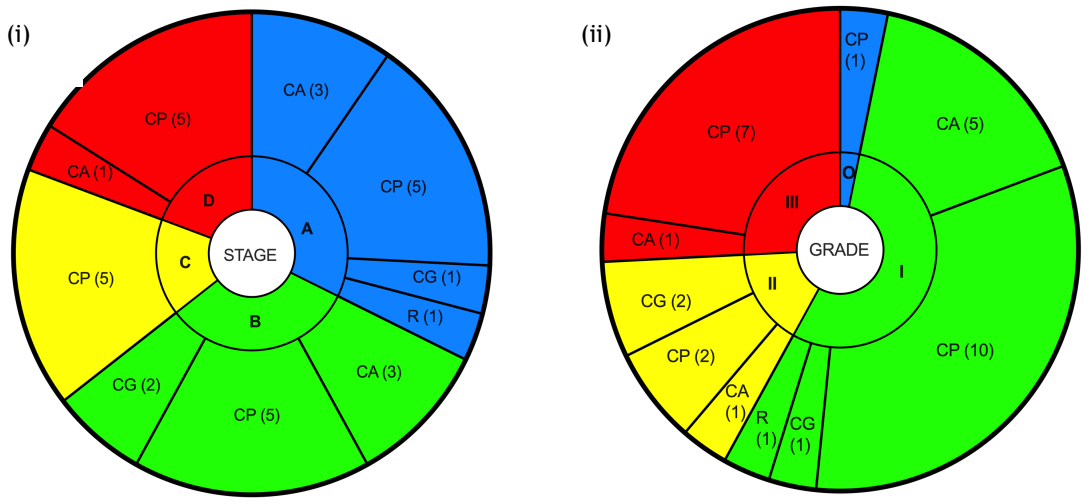


Figure 5.5 Fungal species identified from different (i) stages (ii) grades. A total of 31 fungal species were identified by MALDI-ToF. All isolates were yeast-like organism indicated as CA (C. albicans), CP (*C. parapsilosis* complex), CG (*C. glabrata*) and R (*Rhodotorula mucilaginosa*). Number in bracket represents the number of isolates. * One isolate was identified as C. metapsilosis and was grouped together under *C. parapsilosis* complex.

5.4.4 Biofilm variation between strains and susceptibility profiles

All the fungal isolates identified in Figure 5.5 were characterised for biofilms and antifungal profiles (Table 5.2). Biofilm formation varies among the isolates (Figure 5.6). *C. albicans* displayed the most robust biofilms compared to other *Candida* species.

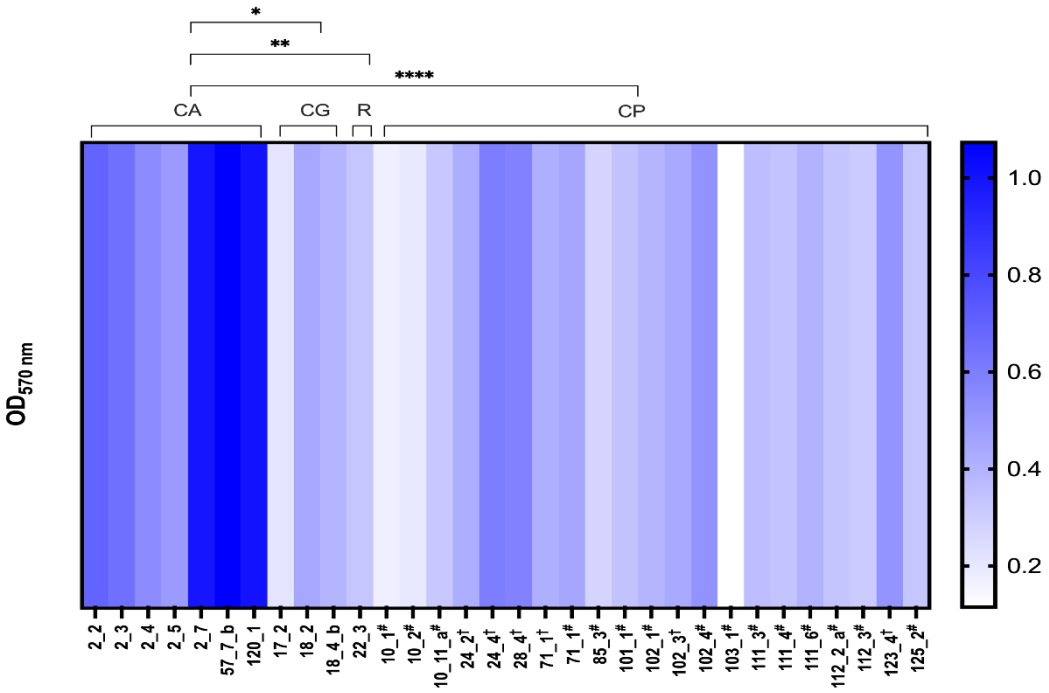
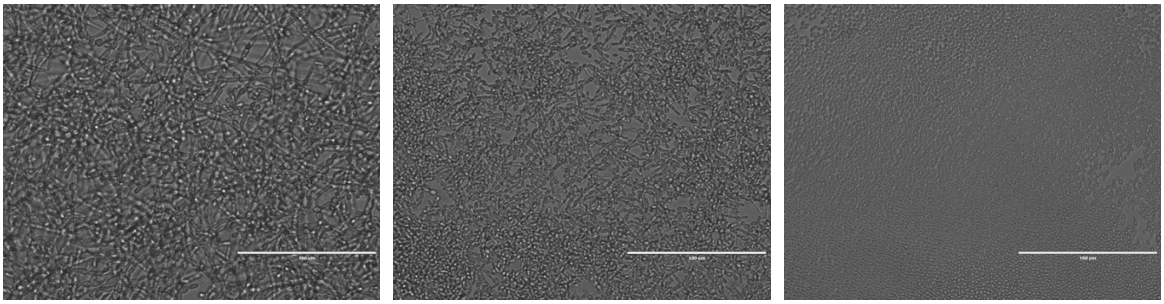


Figure 5.6 Heterogenous biofilms formed by fungi. # and † indicate the smooth and wrinkled phenotypes of *C. parapsilosis* complex isolates. Biofilms formed by *C. albicans* was significantly different (adjusted p-value from Kruskal-Wallis test) than *C. glabrata* (*p<0.0316), *Rhodotorula mucilaginosa* (**p<0.0014) and *C. parapsilosis* complex isolates (****p<0.0001).

Microscopic imaging of *C. albicans* biofilms shows very dense interconnected hyphae after 24 hours of incubation. On the other hand, *C. parapsilosis* biofilms were loosely covered at the bottom of the microtiter plate with the presence of pseudohyphae and blastospores. *C. glabrata* biofilms consisted only of blastospores without any hyphae (Figure 5.7).



(i) (ii) (iii)

Figure 5.7 Morphology of biofilms formed by *Candida* isolates *C. albicans* (i), *C. parapsilosis* (ii) and *C. glabrata* (iii) biofilms after being grown for 24 hours in RPMI medium.

Antifungal susceptibility profiles were tested against all planktonic and sessile forms of the fungal isolates. Table 5.2 summarised the planktonic and the sessile MIC against Fluconazole, Caspofungin and Amphotericin B. For planktonic MIC, *Candida* species fell within the range of ≤ 0.125 – 8 µg for Fluconazole, 0.25 – 4 µg for Caspofungin and up to 1 µg for Amphotericin B. In contrast, all biofilms were greater or equal to the highest tested concentration for Fluconazole (128 µg/mL).

Table 5.2 Antifungal susceptibility profiles of fungal isolates. Planktonic MIC (PMIC) and sessile MIC at 90% inhibition (SMIC) of fungal species isolated from the wounds against three conventional antifungals; Fluconazole (FZ), Caspofungin (CAS) and Amphotericin B (AMB). Values represent the median from three replicates. Growth inhibition was visually read except for Fluconazole against *C. albicans* isolates that were read by spectrophotometer at 530 nm wavelength for 50% inhibition due to the trailing effect. Symbols # and † indicate smooth and wrinkled phenotype of *C. parapsilosis* complex isolates respectively.

Patient	Fungi	PMIC (µg/mL)			SMIC at 90% (µg/mL)		
		FZ	CAS	AMB	FZ	CAS	AMB
2_2	*C. albicans*	0.5	2	1	>128	64	2
2_3	*C. albicans*	0.5	2	1	>128	64	1
2_4	*C. albicans*	≤ 0.125	0.5	≤ 0.125	>128	64	1
2_5	*C. albicans*	≤ 0.125	0.25	≤ 0.125	>128	64	1
2_7	*C. albicans*	0.25	0.5	≤ 0.125	>128	1	2
10_1	*C. parapsilosis*#	1	4	1	>128	>128	4
10_2	*C. parapsilosis*#	0.5	4	1	8	>128	1
10_11_a	*C. parapsilosis*#	2	1	≤ 0.125	>128	>128	>128
17_2	*C. glabrata*	8	0.5	0.25	>128	>128	>128
18_2	*C. glabrata*	4	0.5	≤ 0.125	>128	2	8
18_4_b	*C. glabrata*	4	0.5	≤ 0.125	>128	>128	>128
22_3	*R. mucilaginosa*	>64	16	0.5	>128	>128	>128
24_2	*C. parapsilosis*†	1	1	≤ 0.125	>128	>128	0.5
24_4	*C. parapsilosis*†	2	4	1	>128	>128	64
28_4	*C. parapsilosis*†	2	4	1	>128	>128	4

*Table 1.2 continued

57_7_b	*C. albicans*	0.25	0.25	≤ 0.125	>128	64	2
71_1	*C. parapsilosis* [#, †]	1[#],2[†]	1 [#, †]	≤ 0.125 [#, †]	>128 [#, †]	>128 [#, †]	4[#], 0.5[†]
85_3	*C. parapsilosis*[#]	1	4	1	>128	>128	16
101_1	*C. parapsilosis*[#]	1	1	≤ 0.125	>128	>128	32
102_1	*C. parapsilosis*[#]	1	1	≤ 0.125	>128	>128	64
102_3	*C. parapsilosis*[†]	1	4	1	>128	>128	>128
102_4	*C. parapsilosis*[#]	1	1	≤ 0.125	>128	>128	4
103_1	*C. metapsilosis*[#]	2	2	1	>128	>128	4
111_3	*C. parapsilosis*[#]	1	1	≤ 0.125	>128	>128	32
111_4	*C. parapsilosis*[#]	1	1	≤ 0.125	>128	>128	>128
111_6	*C. parapsilosis*[#]	1	4	1	>128	>128	>128
112_2_a	*C. parapsilosis*[#]	1	1	≤ 0.125	>128	>128	>128
112_3	*C. parapsilosis*[#]	1	1	≤ 0.125	>128	>128	2
120_1	*C. albicans*	1	2	1	>128	1	4
123_4	*C. parapsilosis*[†]	1	1	≤ 0.125	>128	>128	4
125_2	*C. parapsilosis*[#]	1	4	1	>128	>128	4

5.4.5 Co-existence of fungi and bacteria in wounds

The dynamic nature of the wounds was further analysed with the co-existence of bacteria from the standard culture and fungi from the enhanced culture. All fungi identified in 5.4.3 were found to co-exist with bacteria (Figure 5.8). Excluding the data reported as 'anaerobes', 'mixed skin' and 'no significant growth', the overall percentage of fungi with gram-positive and gram-negative bacteria were 38.7% and 67.7% respectively. Of the 31 fungi isolated from the enhanced culture, just over two-thirds were found to co-exist with enterics bacteria. *S. aureus* was the most predominant gram-positive bacteria found with fungi (91.7%) while anaerobes were found in about 16.1%.

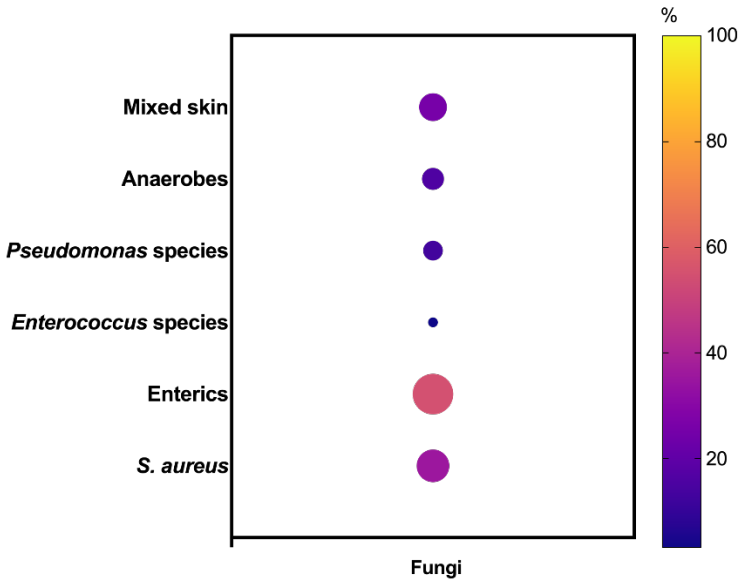


Figure 5.8 Coexistence of bacteria with fungi. Fungi were found to co-exist with gram-positive and gram-negative bacteria including anaerobes. Bacteria and fungi cultures were obtained from standard and enhanced culture data, respectively.

5.5 Discussion

As summarised in Chapter 1, vast species of fungi were identified from diabetic wounds such as *Candida* species and filamentous fungi which include dermatophytes and other ubiquitous mould like *Aspergillus* and *Fusarium* species. These studies had proven the evidence of fungi aetiology from infected wounds with most of them being diagnostic in nature and warranted treatment. It is not surprising to see culture was used in most of these studies as it was considered a gold standard in microbiology labs. Moreover, culture can also be a stand-alone test to diagnose fungal disease from sterile sites according to EORTC/MSG consensus criteria (Donnelly et al., 2020). Serology markers for example will have to be confirmed with additional investigations for a proven diagnosis. Similarly, lack of standardisation in PCR limits its wide use for confirming fungal disease except when the fungal elements were already seen in tissue. Therefore, an appropriate method is crucial for detecting fungal pathogens for an accurate diagnosis and timely treatment.

In this present study, when comparing different identification methods, no clear benefit of enhanced culture compared to basic culture in detecting fungi was observed. A similar number of samples were detected positive for fungi by both culture methods despite a discordant result for some samples. This could be explained by the nature of the clinical samples that were non-homogenous. On the other hand, ITS showed 31.52% detection of fungi in the wound which was significantly higher than the culture. It is encouraging to compare the work that has been done by Kalan *et al*. who found that about 70% of the DFU were positive for fungal DNA as sequenced using the ITS1 primers (L. Kalan et al., 2016). In a much larger cohort of wound specimens from various wound types including DFU, the incidence was reported to be at 23% (Dowd et al., 2011). ITS detection reported by our study was based on the ITS2 gene and was corrected against the control swabs as fungi were known to be ubiquitous in the environment. While previous studies described many medically important fungi in the wounds, they were lacking in the diagnostic utility for fungal diagnosis. This is because, fungi are ubiquitous and opportunistic therefore detection of various species might not always correlate with infection. In this current study, a total fungal load did not show any significant difference across the grades and stages, however, it highlights that fungal load can remain similar despite the severity of the wounds.

These data need to be interpreted with caution because rDNA copies can vary between fungi and, therefore, may affect the fungal load measured by the ITS. As demonstrated in this study, fungal load tends to be higher by ITS compared to the enhanced culture. This result was expected as a single fungal cell may contain multiple copies of rDNA gene, and the numbers can also vary between species. In silico studies shows a range of 14 – 1442 copies of rDNA found across 91 fungal taxa (Lofgren et al., 2019). In this case, the fungal load quantified from the enhanced culture may offer a better estimate of viable fungal cells. High sensitivity methods such as qPCR may be useful to rule out the presence of fungi. Furthermore, higher fungal load was found to be more correlated with growth by culture indicating the viability potential of the fungi.

Unlike bacteria, the clinical importance of fungi has often dismissed as clinically insignificant. Although a study by Kalan *et al*. (2016) did not find a pattern of consistent fungal species across the subjects tested in their study, *Candida* species was one of the most common to be present. Of these, *C. albicans* remains the most frequently identified fungal species (L. Kalan et al., 2016). However, a closer look at the wound types in a study by Dowd *et al* showed that, DFU was indeed predominated by *C. parapsilosis* in comparison to venous leg ulcers and unhealing surgical wounds which were dominated by *C. albicans* (Dowd et al., 2011). Similarly, *C. parapsilosis* was the most prevalent species isolated from deep tissue of diabetic lower limb wounds (Chellan et al., 2010). Here, the finding supports the evidence of previous observations and was also able to distinguish the smooth and crepe morphotypes of *C. parapsilosis* that were described elsewhere (Laffey & Butler, 2005). It is important to bear in mind that *C. parapsilosis* is commonly found on the skin and within the hospital environment although it is considered less pathogenic than *C. albicans*. However, a recent report on the worldwide emergence of *C. parapsilosis* resistant to Fluconazole is worrying (Daneshnia et al., 2023). Particularly the case with hindsight given the rising cases of *C. auris* during several hospital outbreaks worldwide (J. Chen et al., 2020; Schelenz et al., 2016). Moreover, *C. parapsilosis* was listed as high priority fungal pathogen in WHO FPPL (World Health Organization, 2022). Despite its lower association with virulence to cause disease, further investigations are needed to determine the role played by this species in relation to the wound healing progress.

It is noteworthy, that none of the swabs grew *Malassezia* species despite being regarded as a common coloniser of the skin. This is similar to what has been seen in a previous study which described the foot as less oily compared to other anatomical sites which were more favourable for its growth (L. Kalan et al., 2016). Surprisingly, none of the wound samples grew filamentous mould as reported in previous studies (Chapter 1). It is important to note that culture plates could be contaminated by fungi at any point during the incubation especially if plates were incubated for a prolonged period such as 14 days in this study. Fungi such as *Aspergillus*, *Fusarium* and *Cladosporium* normally grow quickly and suspicion of contamination instead of true case should be considered when interpreting the results. On the other hand, dermatophytes will normally take around two weeks to grow and the incubation time used in this study was sufficient to allow for these fungi to grow.

With culture-guided therapy, clearance of the infective pathogens from chronic wounds was still ineffective suggesting a weak impact of the therapy, persistence, resistance and biofilms formation (James et al., 2008). It has previously been observed extensively that yeast-like organisms such as *Candida* species and filamentous mould are able to form biofilms. Heterogeneity of biofilms has also been demonstrated between clinical strains of *Candida albicans* (Sherry et al., 2014; Vitális et al., 2020). Similarly, fungal isolates in this study demonstrated different capacities at forming biofilms. *C. albicans* biofilms were found to be the most robust compared to the ones formed by other *Candida* species. Also, worth noting that, smooth and wrinkled morphotypes of *C. parapsilosis* were significantly different in biofilms biomass ($p = 0.0002$) with wrinkled morphotypes showing higher biomass than the smooth. A similar observation has been reported elsewhere that smooth morphotypes were the least robust biofilms former (Laffey & Butler, 2005). This means that heterogeneity in biofilm formation is an important factor that determines the clinical outcome.

Persistence isolation of the same *Candida* species from the repeat wound sampling of patients in this study may also indicate the tolerance attribute of the biofilms. Biofilms are known to be harder to treat as this can be explained by an increase in sessile MIC obtained from each individual fungal isolate treated with conventional antifungals. However, this data could not ascertain whether these fungi would behave the same way in polymicrobial biofilms and therefore future

studies would have been more interesting to assess the real-world biofilms from the individual wound with a more diverse microbial composition. A study by Raza and Anurshetru has highlighted that the use of antifungals in fungal-infected wounds has resulted in better prognosis (Raza & Anurshetru, 2017). The importance of biofilms has also led to biofilms-based wound care management which was shown to be more effective at improving the wound (R. Wolcott, 2015b).

As far as polymicrobial biofilms are concerned, the co-existence of bacteria with fungi is inevitable. Such phenonema has been shown in various niches in the human body, including the skin and wound microenvironments. In a study by Dowd et al. (2008) chronicity of the wound infection was driven by multispecies instead of single-species bacteria (Dowd, Wolcott, et al., 2008) and it was apparent that fungal grew alongside other bacteria as none of the fungi were mono-isolated in this study. However, pure fungal isolation from wounds has also been reported from previous study of mainly *Candida* species (Mlinariæ-Missoni et al., 2005). This study however did not attempt to culture for anaerobic bacteria therefore unable to rule out the true absence of bacteria as anaerobes were also found to co-exist with fungi in this present study. Here, *Candida* species were found to co-exist with mostly enteric bacteria as similarly reported in another study (Raza & Anurshetru, 2017). This may not be surprising as their interaction has been commonly described in the gastrointestinal tract (F. Zhang et al., 2022). The resemblance of this relationship beyond niches may suggest a 'healthy' network between them. This data suggests an important point to consider when modelling a mixed-species biofilm in a chronic wound. Apart from enteric bacteria, *S. aureus* is the next common bacteria followed by *Pseudomonas* species and anaerobes to co-exist with *Candida*. It has been demonstrated that *C. albicans* has a synergistic role in the growth of *S. aureus* by way of providing surface area for the bacteria to attach allowing for further dissemination into the host (Peters et al., 2010; Schlecht et al., 2015). On the other hand, observations obtained in a retrospective study of burn wounds by Gupta et. al (2005) has found that the absence of *Candida* species is apparent when *Pseudomonas* species are present either alone or with other bacterial species (Gupta et al., 2005). A limited group or species of bacteria were identified alongside fungi in this study. It seems possible that standard culture may underrepresent the vast species of bacteria due to many limiting factors such

as selective growth conditions for bacterial growth. Therefore, microbiome study by sequencing may offer a comprehensive composition of the microbial populations involved in pathogenicity of a chronic wound.

Overall, this study strengthens the evidence of fungi in DFU, highlighting *Candida* species as an important fungal component in chronic wounds with isolates capable of forming biofilms. The use of enhanced culture may not be necessary when *Candida* infection is suspected as they will usually grow on standard microbiological media. Moreover, a more sensitive method such as qPCR may suggest a beneficial option for samples with low fungal burden. The relevance of enteric bacteria when fungi are present is clearly supported in this study and should be considered when developing a chronic wound biofilm model for studying non-healing wounds. The diversity of the bacteria that were present in this study may be restricted due to the limitation of the culture method, but nevertheless observations in interkingdom interactions in the wound bed are similar to previous publications. Biofilm formation between isolated fungi and their bacterial counterparts were not addressed in this chapter, which would make for logical follow up experiments. Further work on investigating the bacterial and fungal interactions within biofilms may offer a better understanding of their co-existence in chronic wounds.

Chapter highlights:

Candida species are the most common fungi in diabetic foot ulcers with the ability to form biofilms.

Enhanced culture has a similar performance to standard culture in growing *Candida*.

Bacterial populations co-exist with fungi in chronic wounds. Enteric bacteria are the most common bacteria identified with fungi in DFU followed by *S. aureus*.

6 Modelling the antimicrobials impact on real world biofilms

6.1 Introduction

In 2014-2015 up to £962 million was estimated to cover the healthcare cost of managing diabetic foot ulcer (DFU) and amputation by the NHS England (Kerr et al., 2019). Among the first line of antibiotics used to treat mild to severe DFU includes Flucloxacillin, Clindamycin, Gentamicin, with additional antibiotics, such as Vancomycin when MRSA is suspected (Barwell et al., 2017). Empirical therapy based on the culture and sensitivity results following planktonic single species MIC testing might result in switching to antibiotics that are more appropriate for the isolated pathogen. However, a consortium of microbes is often found in the wound, existing as polymicrobial biofilms that are inherently tolerant to antibiotics (Dowd, Wolcott, et al., 2008).

In addition, diabetic patients are often found with poor blood circulation which limits the distribution of the antibiotics to the infected wounds (Lipsky, 2004). The low concentration of the antibiotics in the tissue of DFUs is evidenced compared to the serum level concentration (Crowther et al., 2021; Lipsky, 1999). Furthermore, the emergence of antibiotic resistant bacteria in DFU has also become a challenge in managing the infection (Caruso et al., 2021).

Moreover, the presence of fungi in DFU is also important to consider as they are not affected by antibiotics (E. Townsend et al., 2017). This is concerning, as late treatment could exacerbate the wound condition leading to lower limb amputation, and longer ulcer duration is likely to result in fungi being present (Sanniyasi et al., 2015). Therefore, a combination treatment might result in improved pathogens elimination leading to a better prognosis.

A simple wound biofilm model with predetermined composition of bacteria very often consists of pathogenic species such as *S. aureus* and *P. aeruginosa* (Short et al., 2023). While a simple model is useful in terms of observing a direct effect of treatment, however, in reality, wound microbial composition is much more complex involving multi-species interaction which in turn results in a more robust protection from antimicrobials (K. W. K. Lee et al., 2014). Furthermore, the interaction between different species might influence the individual burden of each microbiota (K. W. K. Lee et al., 2014), therefore the abundance of every member in the community may not be the same as normally represented by a

simple biofilm model. In other words, testing on a subset of a predetermined model lacks a true representation of microbes that exist in the wound bed. Thus, assessing the antimicrobial effect on a complex model is more realistic and representative of the actual microbial burden of the wound. The real-world model is anticipated to capture the true diversity of the community to simulate the actual wound.

6.2 Hypothesis and aims

It is hypothesised that wound biofilms, with their complex microbiome and mycobiome, do not respond to antimicrobial therapy in a predictable manner. Yet, most studies assess antimicrobial therapy using standard mono-species planktonic and biofilm models. This chapter intends to model the impact of antimicrobial treatment on chronic wound microbiome by developing real-world biofilms directly from the wound samples and monitoring changes to the composition using Oxford Nanopore Sequencing. It is the intention to demonstrate dynamic changes in the wound microbiome are influenced by the choice of antimicrobial with minimal impact on the resolution of the infection.

6.3 Materials and methods

6.3.1 Assessment of biofilm formation

Prior to assessing the impact of antimicrobials on the biofilms, an initial evaluation was carried out to assess the ability of biofilms to form when directly grown from the clinical samples collected and described in Chapter 2. Wound swabs stored in 2 mL of glycerol at -80℃ were thawed at room temperature before processing. Four swabs from patient numbers 7, 16, 102 and 120 were selected for this initial assessment. These samples were selected as they contained a range of microbes, including mixed enterics, mixed skin, *S. aureus*, *P. aeruginosa* and *C. albicans* to represent diverse wound microbiota. To establish a suitable medium for growing the wound biofilms, two types of media were used. The first medium was RPMI/THB as previously used which has been shown to support the growth of both bacterial and fungal (Alshanta et al., 2022; Montelongo-Jauregui et al., 2016), and the second medium was wound-like media which contains Bolton-broth medium (70.5%), horse serum (20%) and horse blood (0.5 %), as previously described (Sun et al., 2008). Initially, swabs in glycerol were vortex-mixed before removing 200 uL of the glycerol and mixed into 800 µL of PBS as a stock inoculum. Subsequently, the stock was diluted in 1:100 in either RPMI/THB or Bolton broth medium and was inoculated into 24-well plates. The plates were incubated in 5% CO_2 at 37℃ for 72 hours for biofilms to grow. Upon incubation, the supernatant was discarded, and biofilm formation was assessed using a crystal violet assay, as described in Chapter 5. Blank or negative controls consisted of media only.

6.3.2 Characterisation of biofilms from selected samples

Based on the initial assessment performed above, biofilms were grown in RPMI/THB medium for all the selected wounds. The wound swabs selected for the antimicrobial impact assessment were from patient 2 of visits 2, 4, 7 and 9. These samples were collected as they were collected from the same patient over a period of wound progress and this is the only patient that was clinically treated with antifungal, Fluconazole. Moreover, multiple samples from this patient will allow comparison of the dynamic of the microbiome upon treatment. Biofilms were grown and assessed with CV assay as described above.

6.3.3 Biofilms treatment with antibiotic and antifungal

Biofilms were treated with Flucloxacillin (Sigma-Aldrich, UK) and Fluconazole (Sigma- Aldrich, UK) at the concentrations of 0.5 µg/mL and 8 µg/mL, respectively. These concentrations were selected to mimic the therapeutic level of these antimicrobial agents in human tissue (Crowther et al., 2021). A combination of both treatments at the same concentration was also tested. Flucloxacillin and Fluconazole were initially reconstituted according to the manufacturer protocol and were then diluted to the working concentration in RPMI/THB medium. Following 72 hours of biofilm growth, 500 uL of each treatment was added to the 24-well plates containing biofilms. Plates were incubated in 5% CO_2 at 37℃ for 24 hours. Subsequently, the antimicrobial was removed by pipetting and biofilms scrapped in 1 mL PBS.

To assess the viability of the biofilms after treatment, 500 uL of scrapped biofilms in PBS were added with Propidium Monoazide (PMA) (Biotium Inc, Fremont, USA). Samples were incubated in the dark for 10 minutes to allow uptake of the dye. Next, the cells were exposed to the LED light in PMA-Lite TM LED photolysis device (Biotium Inc, Fremont, USA) for 5 minutes. Samples were stored at -20℃ until ready for DNA extraction. The DNA was extracted following the protocol described in Chapter 3. The overall load of 16S and ITS were quantified using the qPCR with the following primers;

16S: 16S F: 5'-TCCTACGGGAGGCAGCAGT-3' and 16S R: 5'-GGACTACCAGGGTATCTAATCCTGTT -3'

ITS: ITS3 Uni F: 5'-TCGCATCGATGAAGAACGCAGC-3'and ITS4 Uni R: 5'-TCTTTTCCTCCGCTTATTGATATGC –3'

Overview of the work as represented in Figure 6.1.

6.3.4 Nanopore sequencing and microbiome analysis

The extracted DNA was processed for nanopore sequencing using the same protocol as described in Chapter 3. Initially, the extracted DNA were quantified using the NanoDrop ND-1000 spectrophotometer (Labtech International, Ringmer, UK) and standardised to 1 ng/µL followed by DNA amplification using the set of

primers and PCR thermal profile as described in section 3.3.3. The amplified DNA products were then cleaned up with AMPure XP beads and quantified using Qubit™ fluorometer before being standardised to 5 ng/µL for barcoding following the method described in 3.3.4 and 3.3.5. Subsequently, DNA barcoding was performed using the PCR barcoding expansion 1-96 using the thermal profiles as described in 3.3.6. and were cleaned up as previously described, followed by the pooling of the barcoded DNA into a Lo-bind Eppendorf tube with another DNA clean-up. The Ligation Sequencing Amplicons V14 (SQK- LSK114) kit was then used for the end-prep following the protocol described in 3.3.7. Briefly, 1 µL of the DNA control sample (DCS) was added to 49 µL of the cleaned pooled DNA. Then, Ultra II end-prep reaction buffer and Ultra II end-prep enzyme mix were added in 7 µL and 3 µL respectively making a total reaction volume of 60 µL. The reaction was incubated in a thermocycler for 5 minutes each at 20 ℃ and 65 ℃ followed by the addition of AMPure XP beads and washing with 80% ethanol on a magnet. Next, the pellet was resuspended in Nuclease-free water and the eluate containing the DNA was transferred into a clean Lo-bind Eppendorf tube. The repaired DNA from the end-prep step was then taken forward into the adapter ligation and clean-up step as described in 3.3.8. In this step, the reagents were sequentially added and mixed with NEBNext Quick T4 DNA Ligase and Ligation adapter (LA) at 10 µL and 5 µL, respectively. The final DNA library was obtained by washing with Short Fragment Buffer (SFB) and eluting the pellets with elution buffer (EB). Finally, the library concentration was adjusted to be in a range of 35 – 50 fmol in 12 µL of elution buffer. The DNA library was stored at 4℃ until ready for loading into a flow cell. Flow cell loading and data acquisition were described in 3.3.9 and 3.3.10, respectively. A total of 48 samples were performed in one sequencing run. Sequencing data was analysed for taxonomy composition and diversity using MicrobiomeAnalyst 2.0. Briefly, the files including OTU count, taxonomy table and metadata were uploaded to the website according to the required format. The OTU table was uploaded in .txt while both taxonomy and metadata were in .csv format. Data normalization was performed based on the rarefying to the minimum size of the library. Community profiling and visual exploration were initially carried out on MicrobiomeAnalyst 2.0 and data were then transferred to GraphPad Prism to create high resolution figures.

6.3.5 Statistical analysis

Statistical analyses and graph production were performed using GraphPad Prism (Version 8.4.3; GraphPad Software Inc., La Jolla, CA). Kruskal-Wallis test was used to compare the biofilm formation and total 16S and ITS load (with Dunn's multiple comparison). A p-value of <0.05 was considered as statistically significant.

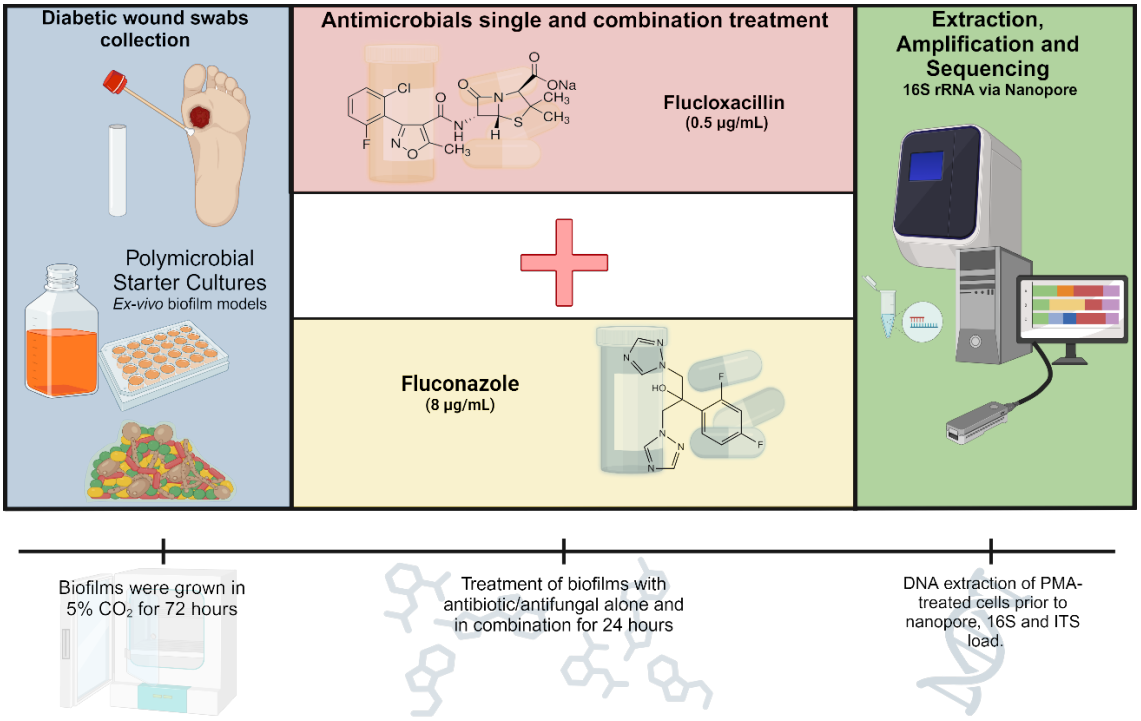


Figure 6.1 Schematic diagram of the antimicrobial treatment on real-world biofilms. Created with BioRender.com.

6.4 Results

6.4.1 Media type influences the biofilm biomass

Initial assessment on the most suitable medium for biofilm formation was evaluated using a wound-like medium that primarily consisted of Bolton-broth, horse serum, blood and RPMI/THB medium. CV assay was used to quantify the biofilms formation after 72 hours from swabs 7, 16, 102 and 120. Swabs 7 and 16 contained *S. aureus* and mixed *Pseudomonas*/anaerobes, respectively. On the other hand, 102 and 120 contained *Candida* species based on enhanced culture (Chapter 5) including mixed skin and mixed enterics, respectively. Figure 6.2 compares the biomass from the CV assay obtained from the two types of media. It can be observed from the data in Figure 6.2 that higher OD values were obtained from samples that contained fungi grown in RPMI/THB compared to the wound-like media. Interestingly, sample 16 has the highest biomass in wound-like media compared to other samples grown in the same media. No significant difference was observed between the media type for sample 7, though notably biofilm formation was significantly lower than in sample 16 in wound-like media. Figure 6.3 provides the CV assay staining images of the respective samples in the two media type, and these data confirm the quantitative data presented.

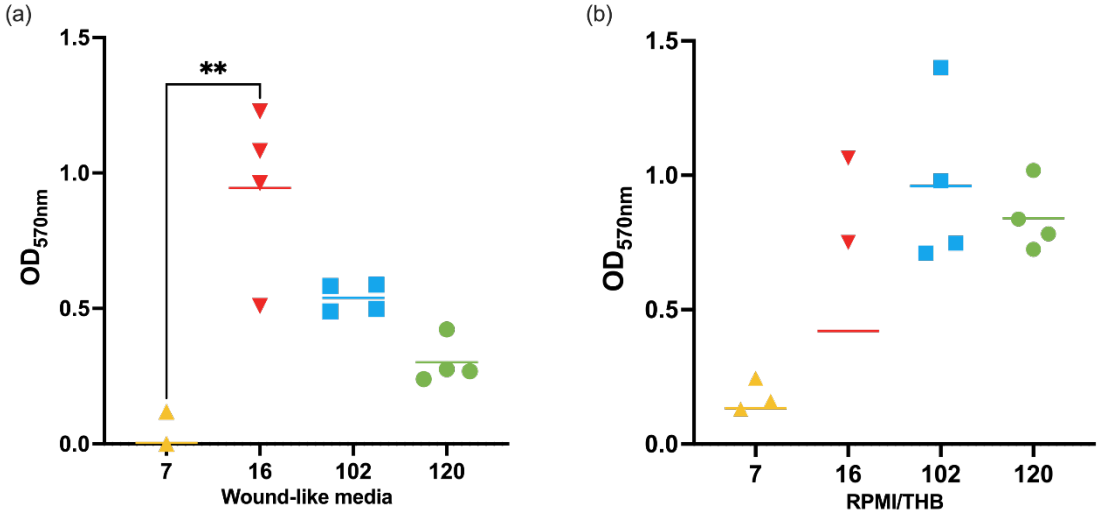


Figure 6.2 Biofilms formation in two different media. Four wound swabs (7, 16, 102 and 120) were grown in either wound-like media **(a)** or RPMI/THB **(b)** for 72 hours in 5% CO_2. Biofilm formed were assessed using crystal violet assay measured at 570 nm wavelength. Data represent each individual replicate with line indicating the mean. ** Adjusted p = 0.0038 based on Kruskal- Wallis with Dunn's multiple comparison test.

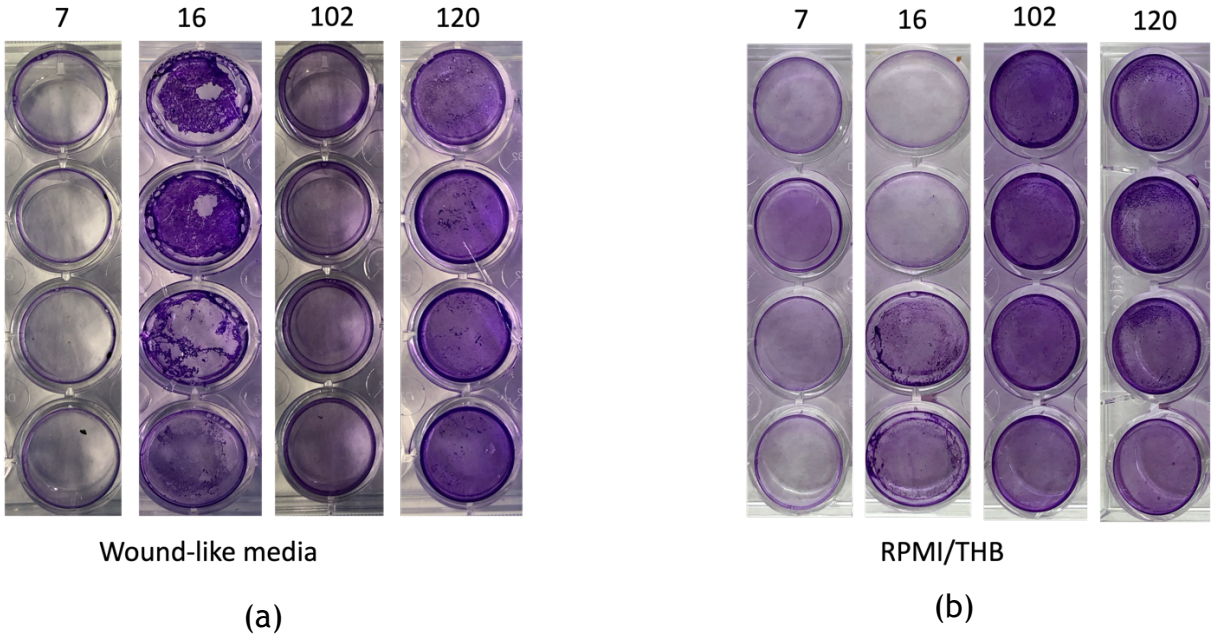


Figure 6.3 Crystal violet stained on individual biofilms. Images taken prior to the de-staining step represent growth in (a) wound-like media and (b) RPMI/THB.

Based on the data from Figures 6.2 and 6.3, RPMI/THB was used as the medium to grow the real-world biofilms, and the assessment of the antimicrobial effects from patient 2 of visits 2, 4, 7 and 9. Before proceeding to examine the antimicrobial impact on the selected samples, biofilm biomass was characterized and presented in Figure 6.4. All biofilms were similar in terms of biomass except biofilms from visits 4 and 7 which shows a statistically significant difference ($p = 0.0194$).

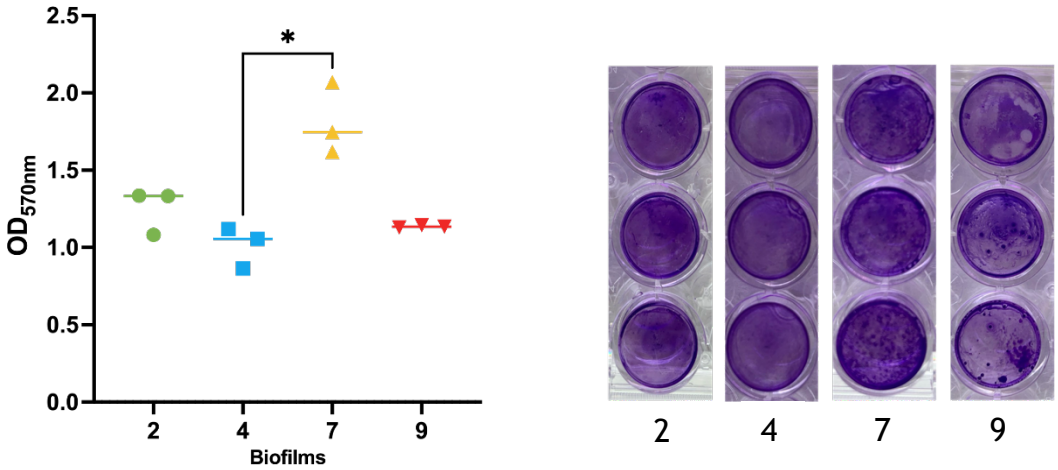
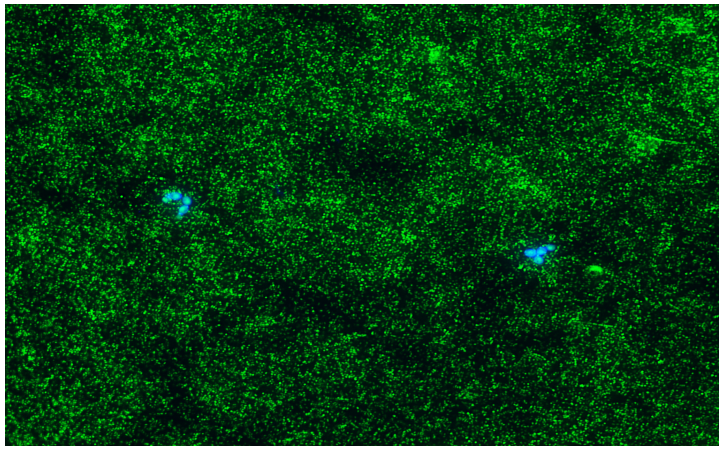


Figure 6.4 Characterising biofilms in RPMI/THB media. Following media selection, wound swabs of patient 2 from visits 2, 4, 7 and 9 were grown in RPMI/THB for 72 hours in 5% CO_2. OD represent the individual values from CV assay with respective stained biofilms images shown on the side. Adjusted * $p = 0.0194$ based on Kruskal- Wallis with Dunn's multiple comparison test.

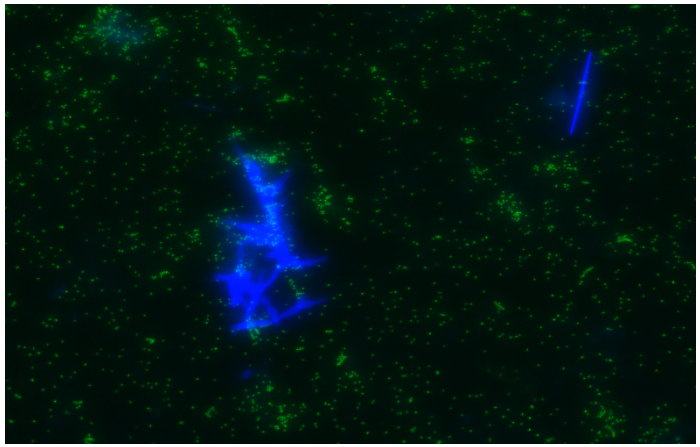
Fluorescent imaging of the biofilms (Figure 6.5) shows the presence of yeast cells (blue) in the biofilm of patient 2 from visits 2, 4, and 7 with bacteria observed in green. Closer inspections of the images show that bacteria were closely attached to the *Candida* in biofilm from visit 4 and 7. Interesting to note the presence of bacteria surrounding the 'mother-cell' of which the hyphae originated from rather than the actual hyphae in biofilm from visit 7. The presence of *Candida* cells was only indicated with the appearance of budding yeast with limited presence of hyphae and pseudohyphae.

(a)



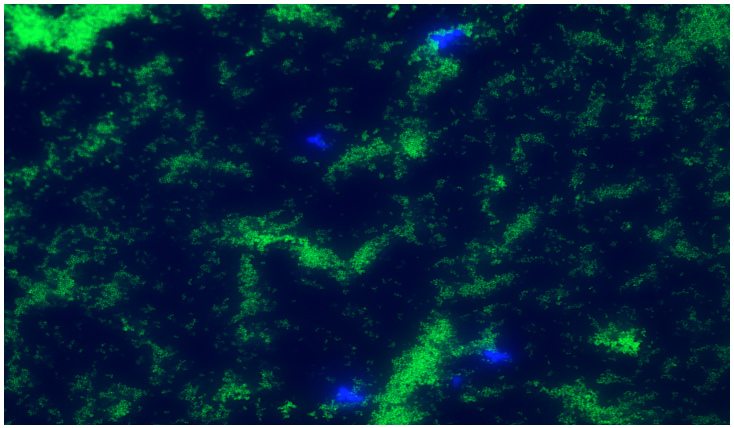
Visit 2

(b)



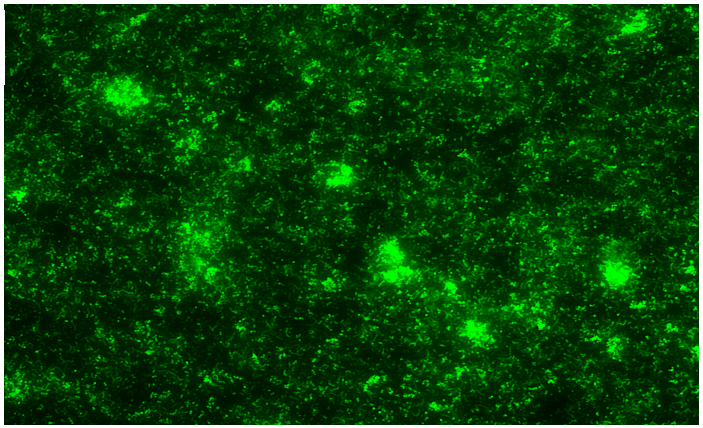
Visit 4

(c)



Visit 7

(d)



Visit 9

Figure 6.5 Biofilms images of patient 2 from different visits. Fluorescence imaging of untreated biofilms stained with SYTO-9 (green) and Calcofluor white (blue) after 72 hours. Biofilms **(a)**, **(b)** and **(c)** show the presence of bacteria (green) and *Candida* cells (blue) and **(d)** only contain bacteria.

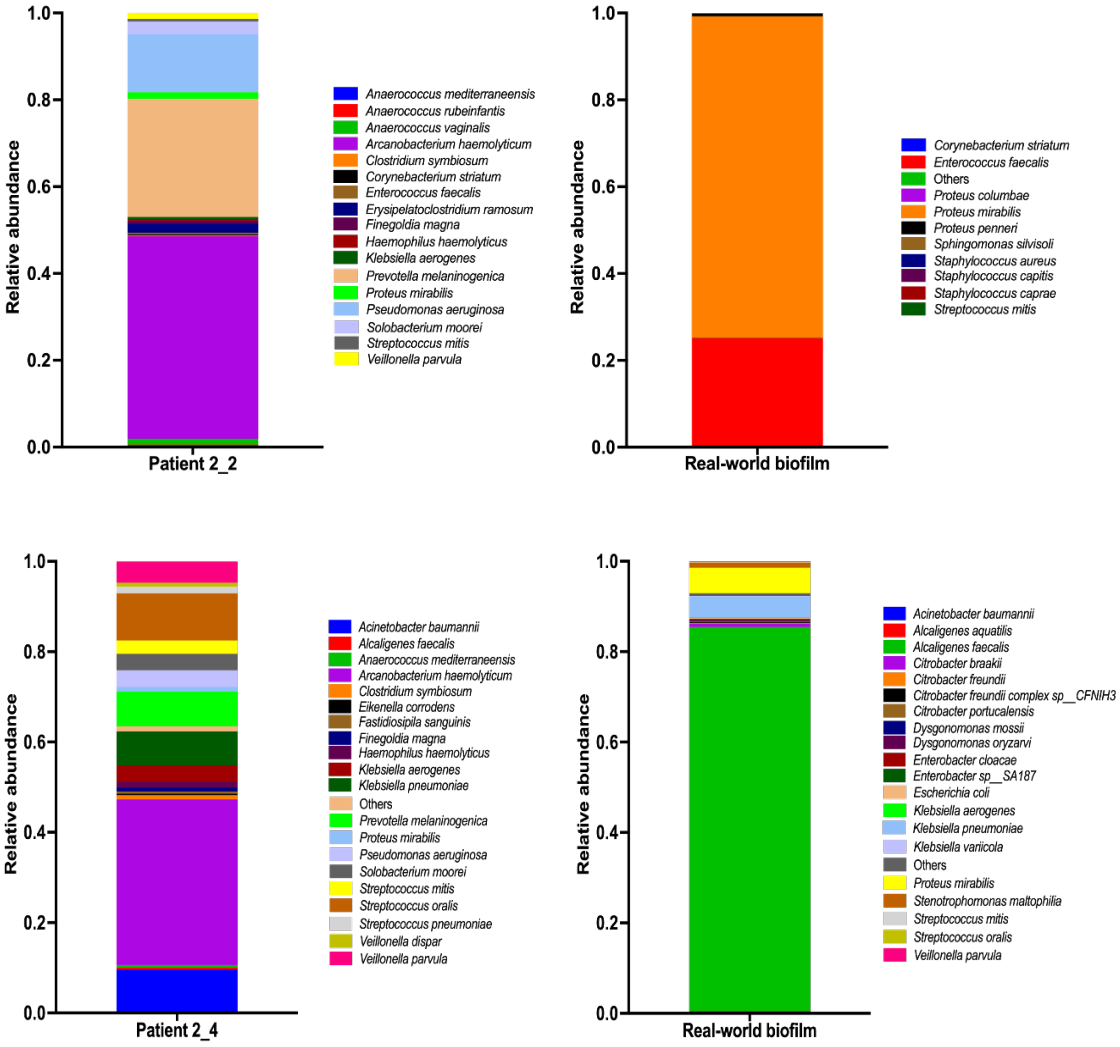
6.4.2 Microbiome differences between patient and real-world biofilm model

Before investigating the impact of antimicrobials on the real-world biofilms model, it is paramount to examine and compare the microbial composition directly from its unaltered wound condition with the current model. The four visits from patient 2 were selected as they were not only representing the chronicity of the wound due to the long period of unhealing condition but also because of the stable pattern of the microbiome between the visits. So, this will dictate the reproducibility of the model by presuming that the same key species will be captured in every biofilm grown from each visit hence allowing for evaluating the impact of antimicrobial treatments on the dynamic of the microbiome. While it is more intuitive when comparing the changes in microbiome based on the pattern of the top common taxa across the treatment groups, however, each patient microbiome in this chapter was scrutinize individually while maintaining the consistency of the microbiome between the visits.

There were a very clear compositional differences between the patient microbiome and the real-world biofilms model. As demonstrated in Figure 6.6, the predominant species in the patient microbiome across all the visits were *A. haemolyticum* and *P. melaninogenica*. However, certain species were only present on certain visits which makes each visit unique. In visit 2, it was clear that the predominant species from the patient were not present in the biofilm model. Instead, the species that were present in the biofilms were contributed by the species that was relatively low abundance in the patient. This includes *P. mirabilis* and *E. faecalis* which represent less than 2% of relative abundance in the patient sample. *P. aeruginosa* which represents 13.38% of the patient microbiome was also not captured in the model. Biofilm from visit 2 consisted of the lowest count of taxa compared to other visits.

As similarly observed in biofilm from visit 4, the relatively low abundance species in the patient microbiome contributed to the most predominant species in biofilm model. This can be seen from the presence of *A. faecalis* which was < 1% abundance in the patient microbiome but constituted to 84.9% in the biofilm model. The persistence of *P. mirabilis* in biofilm from visit 4 was also apparent with 5.6% of relative abundance but with just 1% detected from the patient.

As for biofilm from visit 7, the dominant species consisted of *S. aureus* with 53.9% relative abundance followed by 33.1% of *P. mirabilis*. The presence of *A. baumanii* was very close between the patient and the model with 8.9% and 7% of relative abundance, respectively. Biofilm from visit 9 was very similar to visit 7 with the most abundance species predominated by *S. aureus* and *P. mirabilis* with 56.8% and 37.9% of relative abundance, respectively. However, the abundance of these two species was less than 1% in the patient sample.



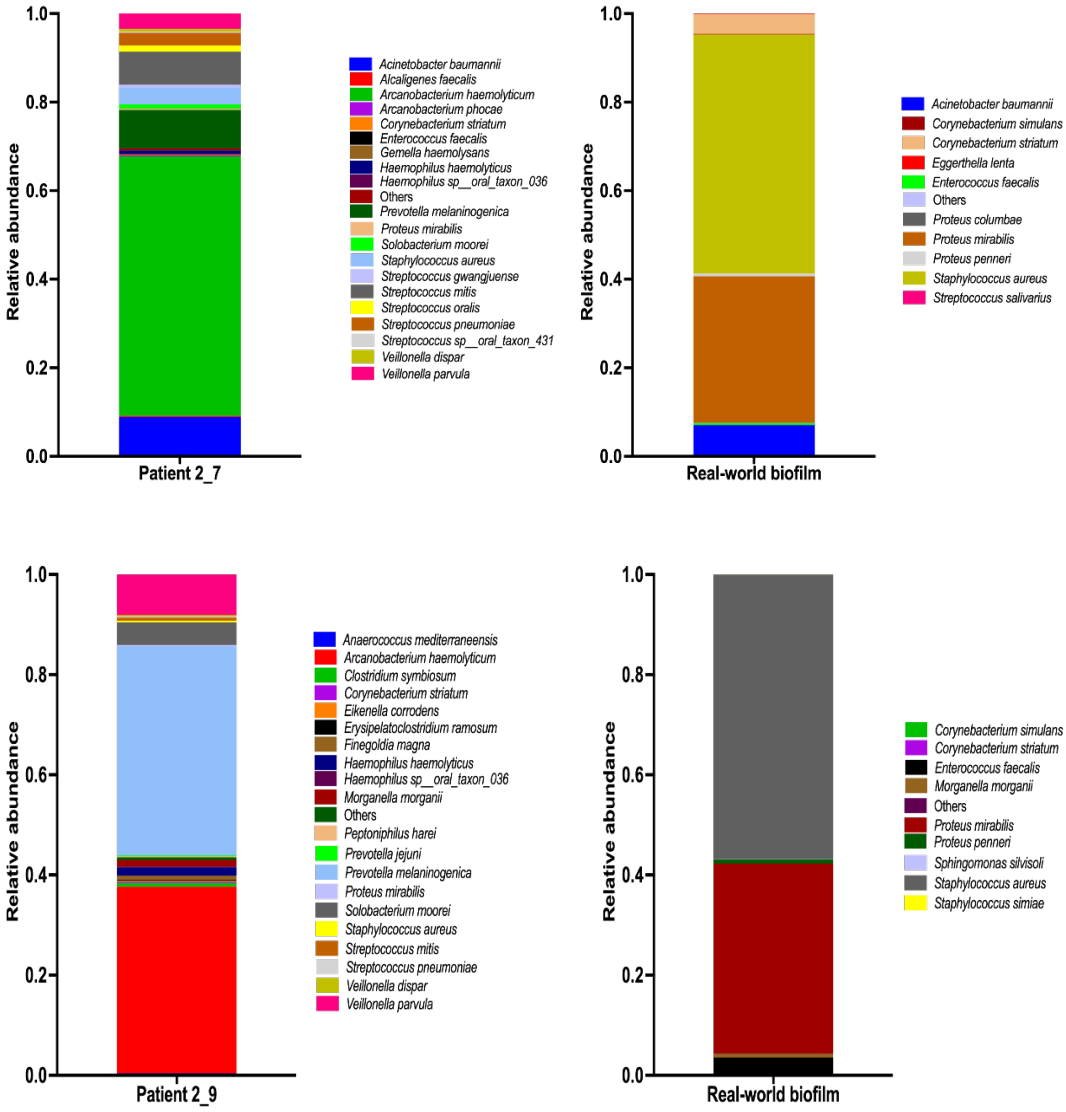


Figure 6.6 Bacterial composition of patients versus real-world biofilm model. Stacked bar plots demonstrate the relative abundance of the top taxa presence in the patients from visits 2, 4, 7 and 9 (left panels) compared to the corresponding models (right panels). The bacterial composition of the real-world biofilm models shows the merged data from three replicates of the untreated group.

6.4.3 Minimal impact of antimicrobial on cell viability

The impact of Flucloxacillin, Fluconazole and the combination of both antimicrobials on the biofilms were quantified by live/dead qPCR using 16S and ITS primers as shown in Figure 6.7. The biofilms were grown from four individual visits (2, 4, 7 and 9) for 72 hours before being treated with 0.5 µg/mL of Flucloxacillin, 8 µg/mL of Fluconazole and the combination of both antimicrobials at the same concentration. Each visit consisted of untreated biofilms that were replenished with fresh medium only. Upon 24 hours of treatment incubation, the untreated biofilms show comparable bacterial load across samples from all visits, ranging from approximately 4×10^7 to 7×10^8. In contrast, the fungal load was much lower only up to 30 CFE/mL in the untreated biofilms. Fungal load appeared to be almost undetected in the untreated biofilms from visit 7. In Figure 6.7, the impact of treatments at reducing the viability of bacteria and fungi was compared to the individual control group from each visit. There were no statistically significant differences ($p > 0.05$, Kruskall-Wallis with Dunn's post-test) observed when comparing the bacterial load upon treatments with any of the antimicrobials. Similarly, all treatment groups do not show a statistically significance difference ($p > 0.05$, Kruskall-Wallis with Dunn's post-rest) in the fungal load for all the visits. However, in visit 2 the fungal load was higher upon treatment with Fluconazole. Similarly, biofilms from visit 7 show higher fungal load in the treatment groups, however, a valid comparison was unable to be made due to the very low viability of fungal cells in the control group. A comparable fungal load was observed in biofilms from visit 4 between the control and the treatment groups. Overall, all treatments do not effectively reduce the total viable bacteria and fungi in the biofilms.

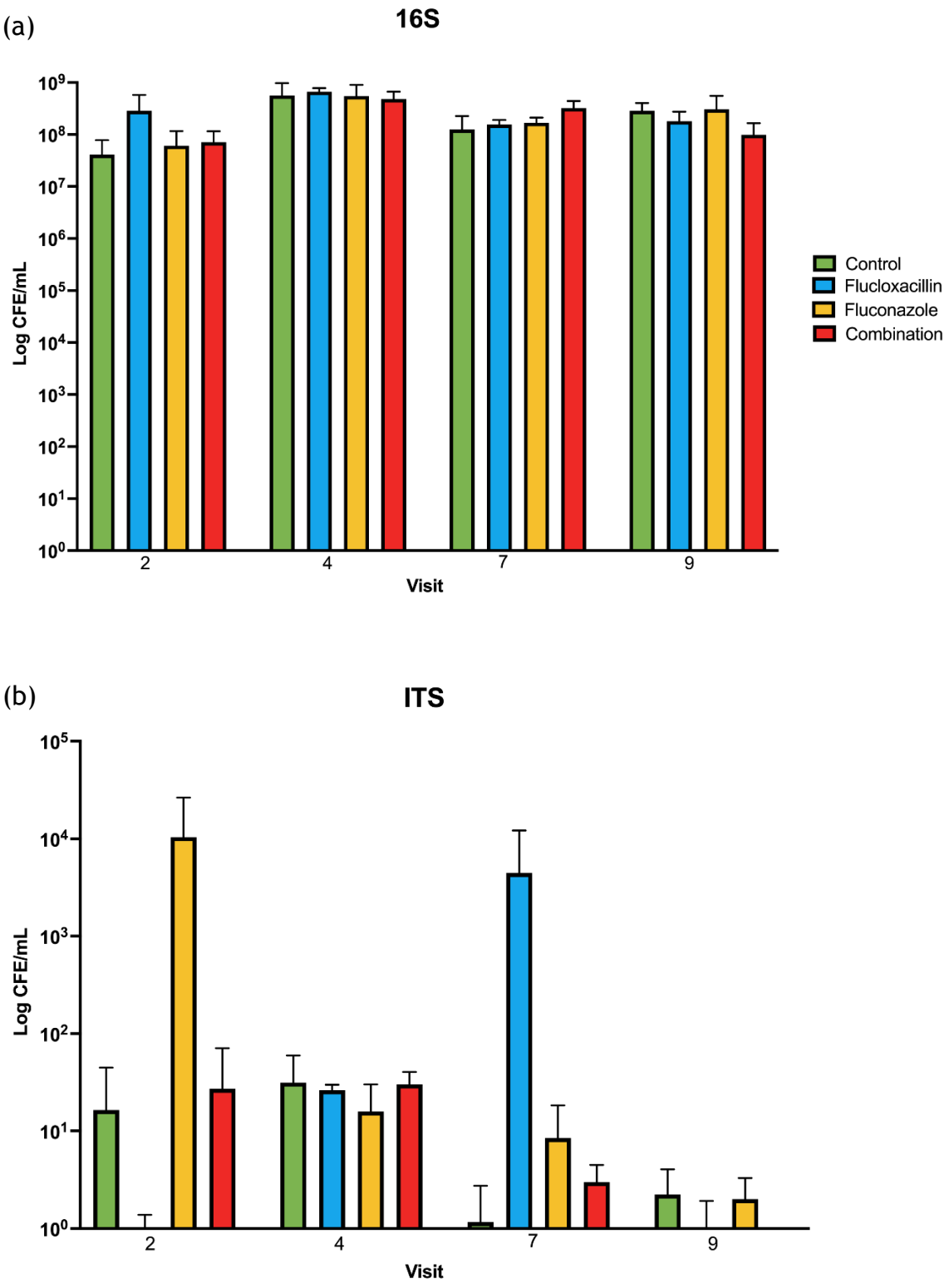


Figure 6.7 Total 16S and ITS load post-treatment. The overall load of 16S **(a)** and ITS **(b)** were quantified by qPCR after treated with Flucloxacillin, Fluconazole and combination with untreated controls for comparison. Each bar represents the mean ± SD from the three replicates. All p values >0.05 based on Kruskal-Wallis test with Dunn's multiple comparison test between each treatment against control.

6.4.4 Impact of treatment on bacterial composition and diversity

The impact of antimicrobial treatments that were obtained from the sequencing were analysed into a relative abundance of bacteria composition and the variation in diversity within and between samples as represented by α and ß – diversity, respectively. Overall, antimicrobial treatments with 0.5 µg/mL Flucloxacillin, 8 µg/mL of Fluconazole and a combination of both treatments did not affect the diversity of the bacterial population. No statistically significance differences were observed as measured by Chao1 and Shannon indices for α-diversity. Similarly, ß-diversity as measured by Bray-Curtis index did not show any statistical differences between the treatment groups.

The biofilms tested can be broadly categorized into predominant gram-negative as from visits 2 and 4 (Figure 6.8, 6.10) and gram-positive as from visits 7 and 9 (Figure 6.12, 6.14). Biofilms from visit 2 were mainly predominated by *P. mirabilis* as the most abundant and *E. faecalis* as the second most abundant species. Although there was no significant difference observed for diversity between treatment groups, combination treatment shows the reduction of diversity as measured based on Chao1 (Figure 6.9) closely followed by treatment with Flucloxacillin compared to the control.

As for biofilms from visit 4, the predominant taxa shifted to *A. faecalis* as the most abundant followed by *P. mirabilis* (Figure 6.10). The biofilms from this visit have higher diversity compared to the previous biofilms from visit 2. When looking at the diversity across treatment groups from visit 4, combination treatment does show a lower diversity than the control and other treatment groups (Figure 6.11). However, there was no statistical significance difference observed.

In visit 7, *S. aureus* and *P. mirabilis* were the two most abundant species in the biofilm (Figure 6.12). In addition, *A. baumanii* was present in all the samples although was relatively low in control 2C1 and 2C2. Similarly, *C. striatum* and *E. faecalis* were detected across all the treatment groups including the control. It is worth noting that there was a reduction of *S. aureus* when treated with a combination in two of the replicate (7XZ1 and 7XZ3). The control from visit 7 was less diverse compared to other treatment groups with no statistical difference observed between them (Figure 6.13).

The same pattern as in visit 7 in terms of the most abundant species was observed in biofilms from visit 9. *S. aureus* and *P. mirabilis* were the most abundant species. Interestingly, *A. haemolyticum* which was observed in the patient microbiome, was detected in samples 9FX3, 9FZ2, 9XZ1 and 9XZ2. However, their presence was relatively low with <1% relative abundance except 2% in sample 9FX3. Diversity across all the treatment groups did not show any statistical difference (Figure 6.15).

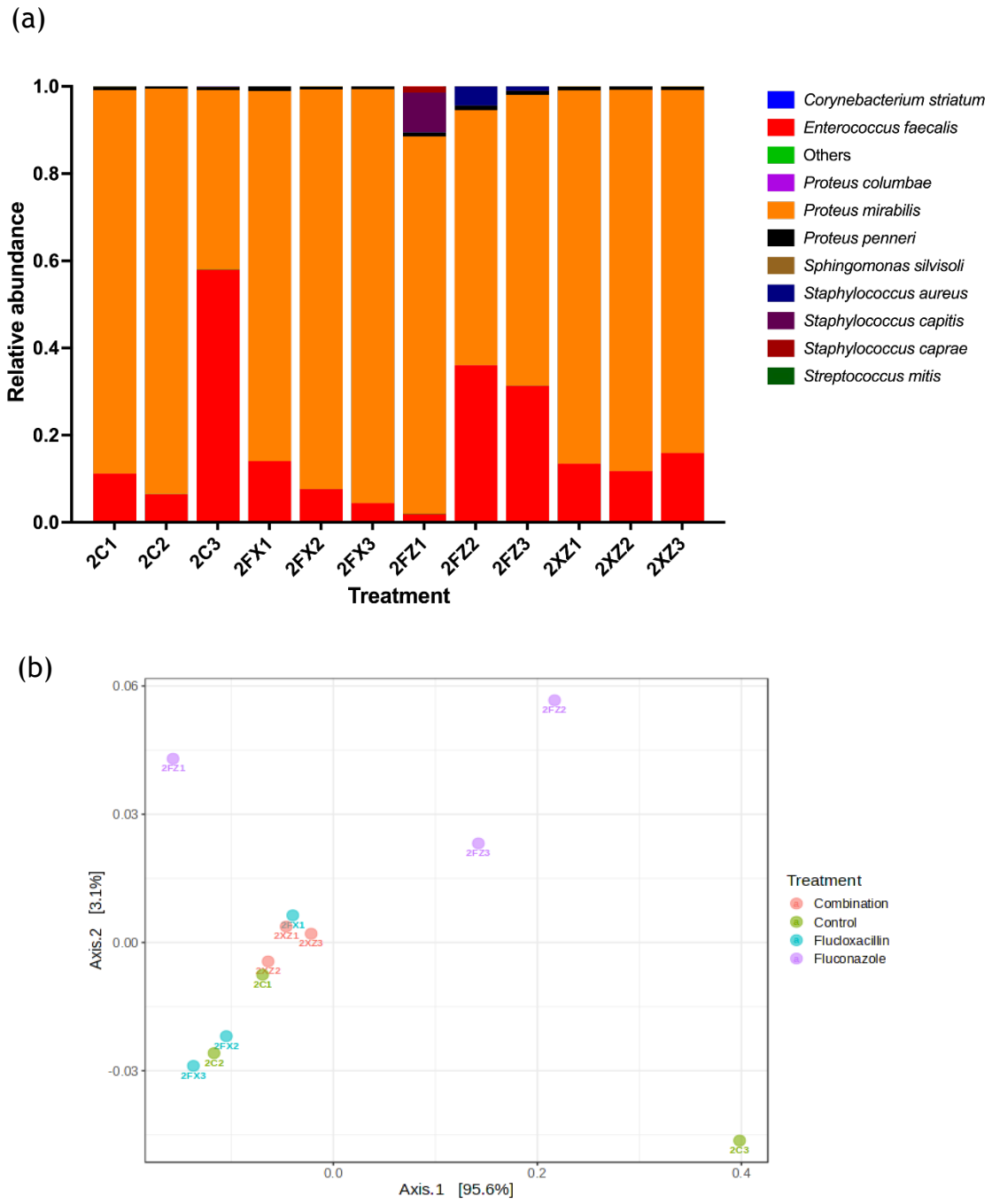


Figure 6.8 Microbiome composition and beta - diversity of treatment groups from visit 2. (a) Stacked bar plots demonstrate the changes in relative abundance of the top most abundance taxa between control (C), and treatments with Flucloxacillin (FX), Fluconazole (FZ), and combination of Flucloxacillin and Fluconazole (XZ). **(b)** PCoA plot with Bray- Curtis dissimilarity show no significant difference ($p > 0.05$) between treatment groups using PERMANOVA with multi-adjustment based on Benjamini – Hochberg procedure.

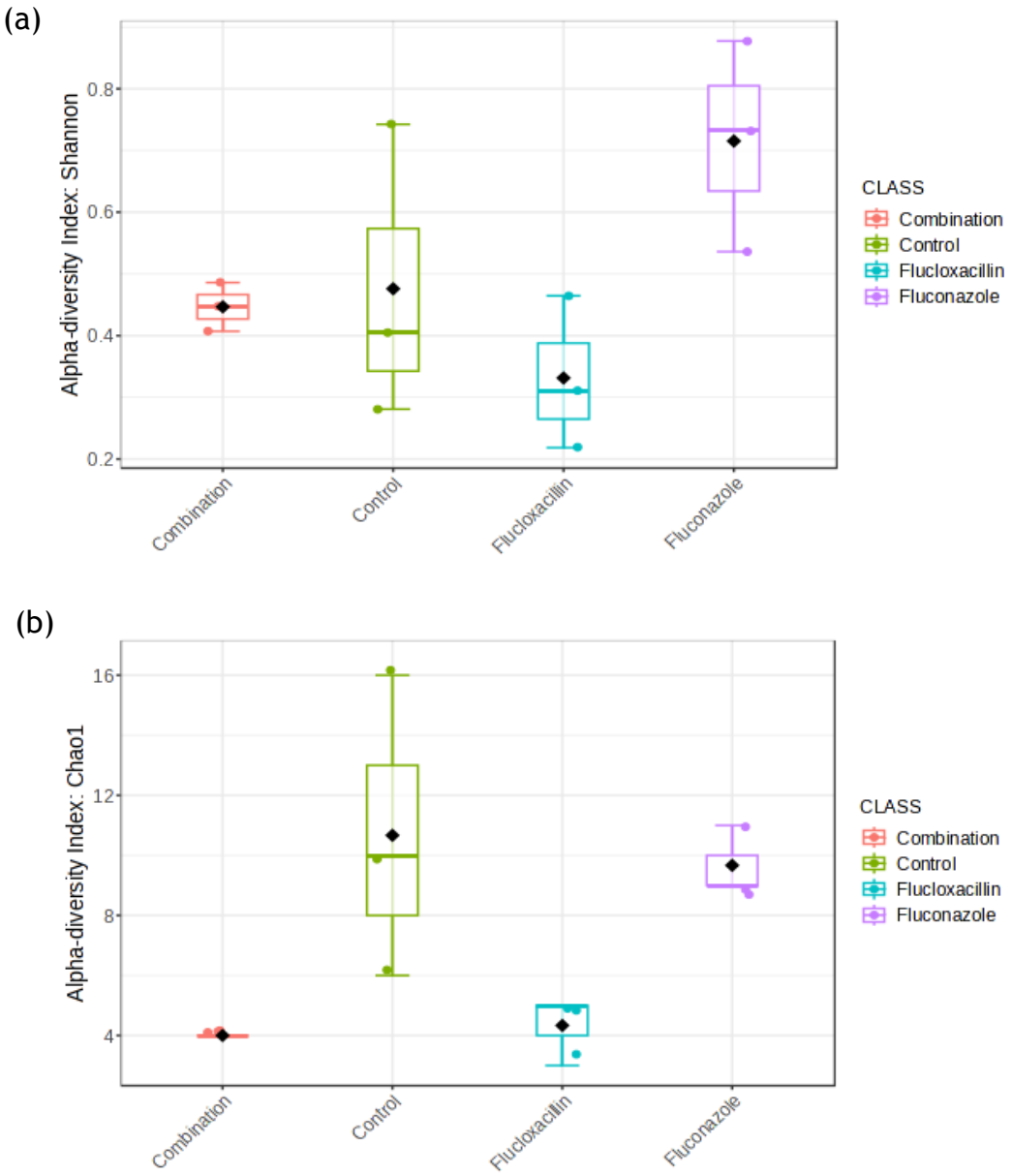


Figure 6.9 Alpha - diversity of treated biofilms from visit 2. Box- plots show the bacterial diversity within the sample of control and treated biofilms as measured using Chao1 **(a)** and Shannon **(b)** diversity indices. No significant differences ($p > 0.05$) were observed for both indices using Kruskal-Wallis with post-host Wilcoxon pairwise comparison with multi-testing adjustment based on Benjamini-Hochberg procedure.

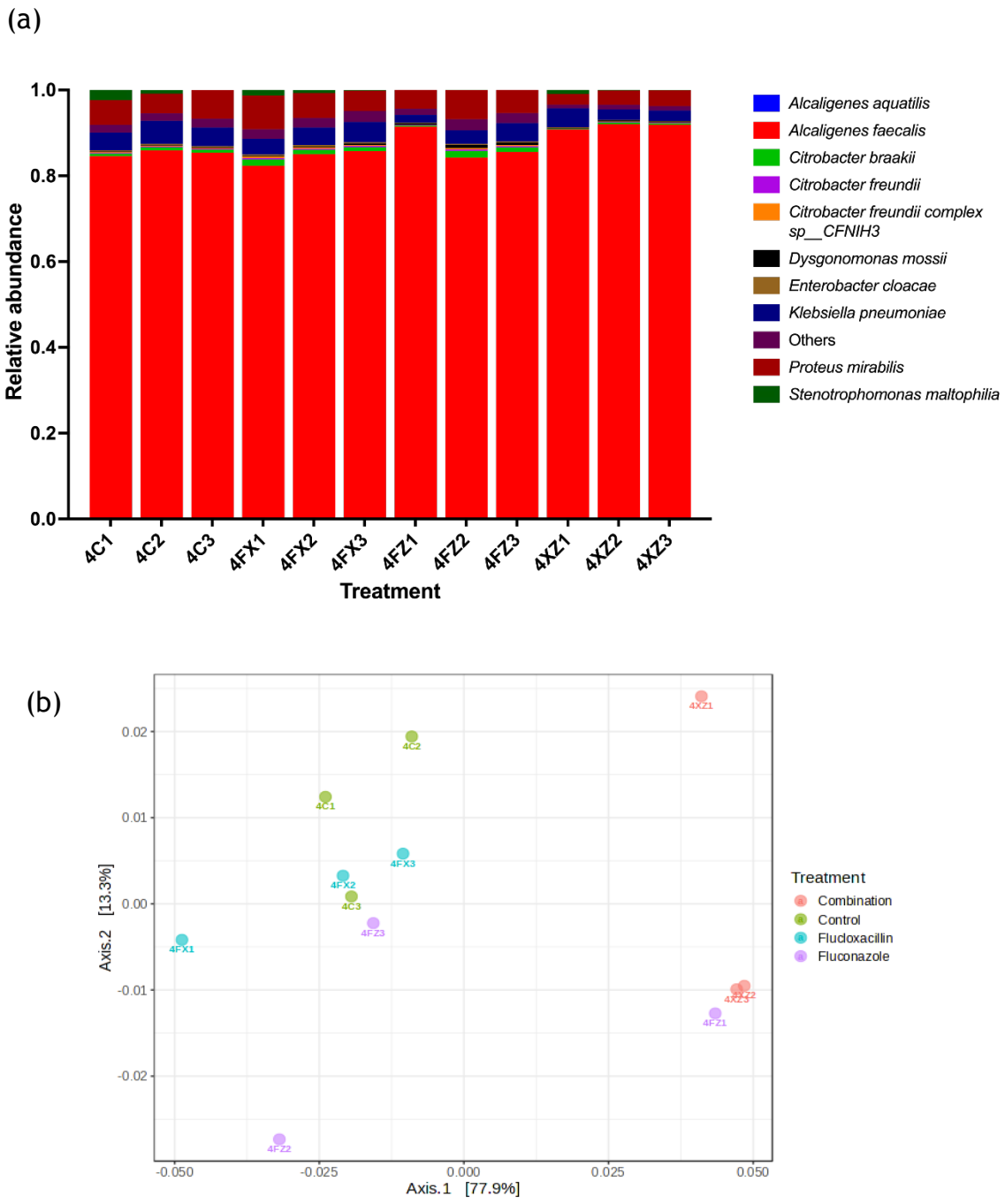


Figure 6.10 Microbiome composition and beta - diversity of treatment groups from visit 4. (a) Stacked bar plots demonstrate the changes in relative abundance of the top most abundance taxa between control (C), and treatments with Flucloxacillin (FX), Fluconazole (FZ), and combination of Flucloxacillin and Fluconazole (XZ). **(b)** PCoA plot with Bray – Curtis dissimilarity shows no significant difference (p >0.05) between treatment groups using PERMANOVA with multi-adjustment based on Benjamini – Hochberg procedure.

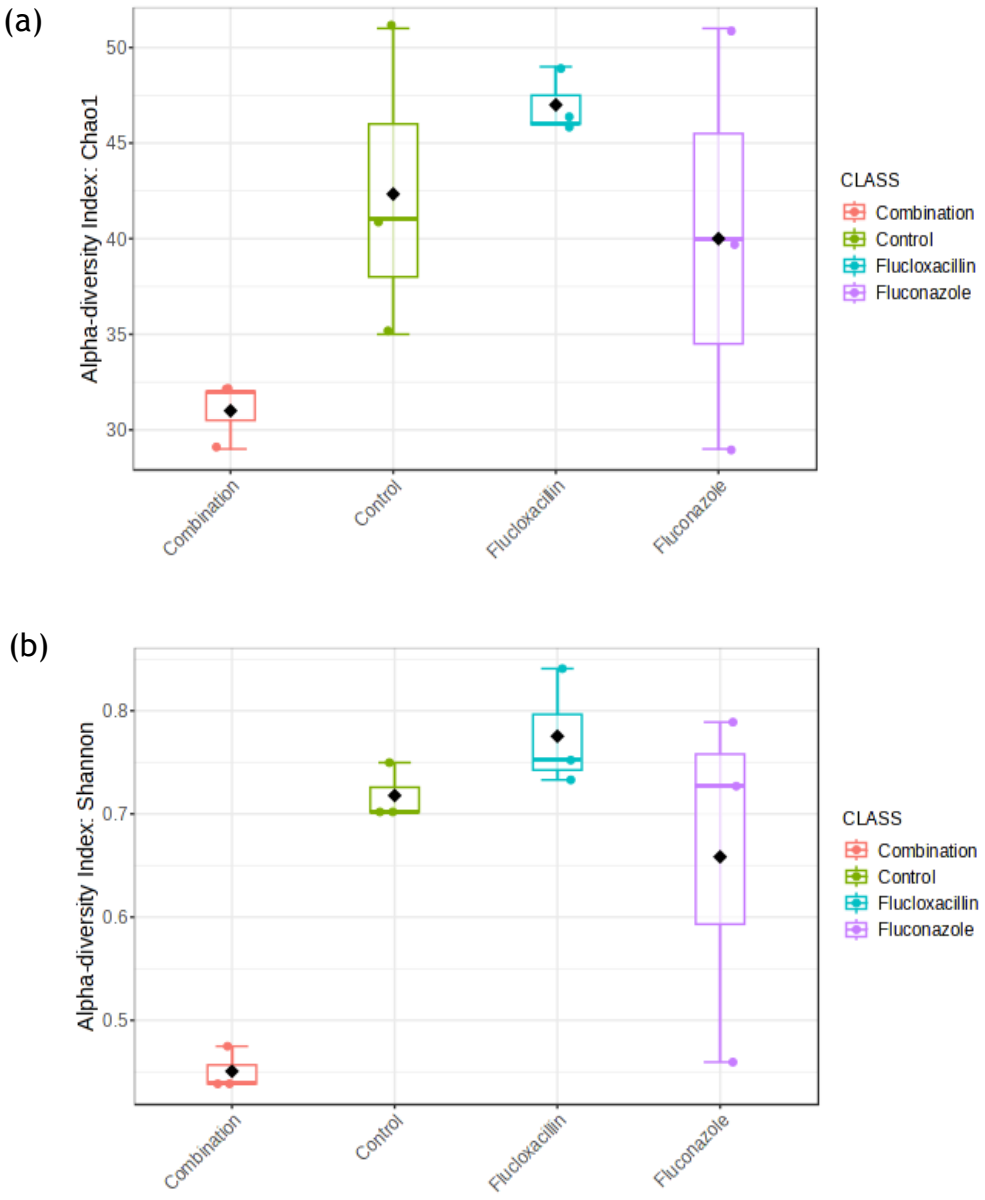


Figure 6.11 Alpha - diversity of treated biofilms from visit 4. Box- plots show the bacterial diversity within the sample of control and treated biofilms as measured using Chao1 **(a)** and Shannon **(b)** diversity indices. No significant differences ($p > 0.05$) were observed for both indices using Kruskal-Wallis with post-host Wilcoxon pairwise comparison with multi-testing adjustment based on Benjamini-Hochberg procedure.

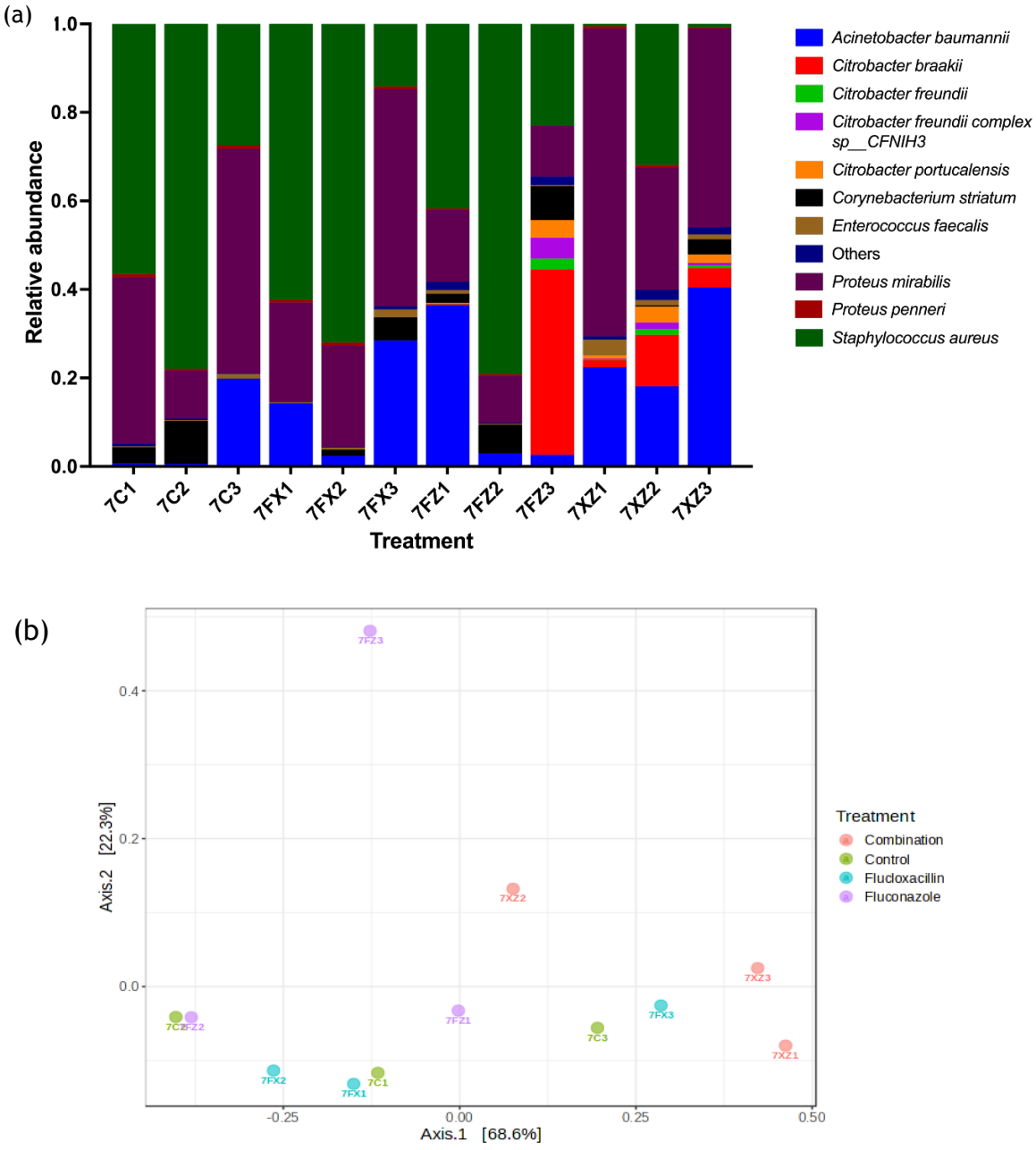


Figure 6.12 Microbiome composition and beta - diversity of treatment groups from visit 7. (a) Stacked bar plots demonstrate the changes in relative abundance of the top most abundance taxa between control (C), and treatments with Flucloxacillin (FX), Fluconazole (FZ), and combination of Flucloxacillin and Fluconazole (XZ). **(b)** PCoA plot with Bray – Curtis dissimilarity shows no significant difference ($p > 0.05$) between treatment groups using PERMANOVA with multi-adjustment based on Benjamini – Hochberg procedure.

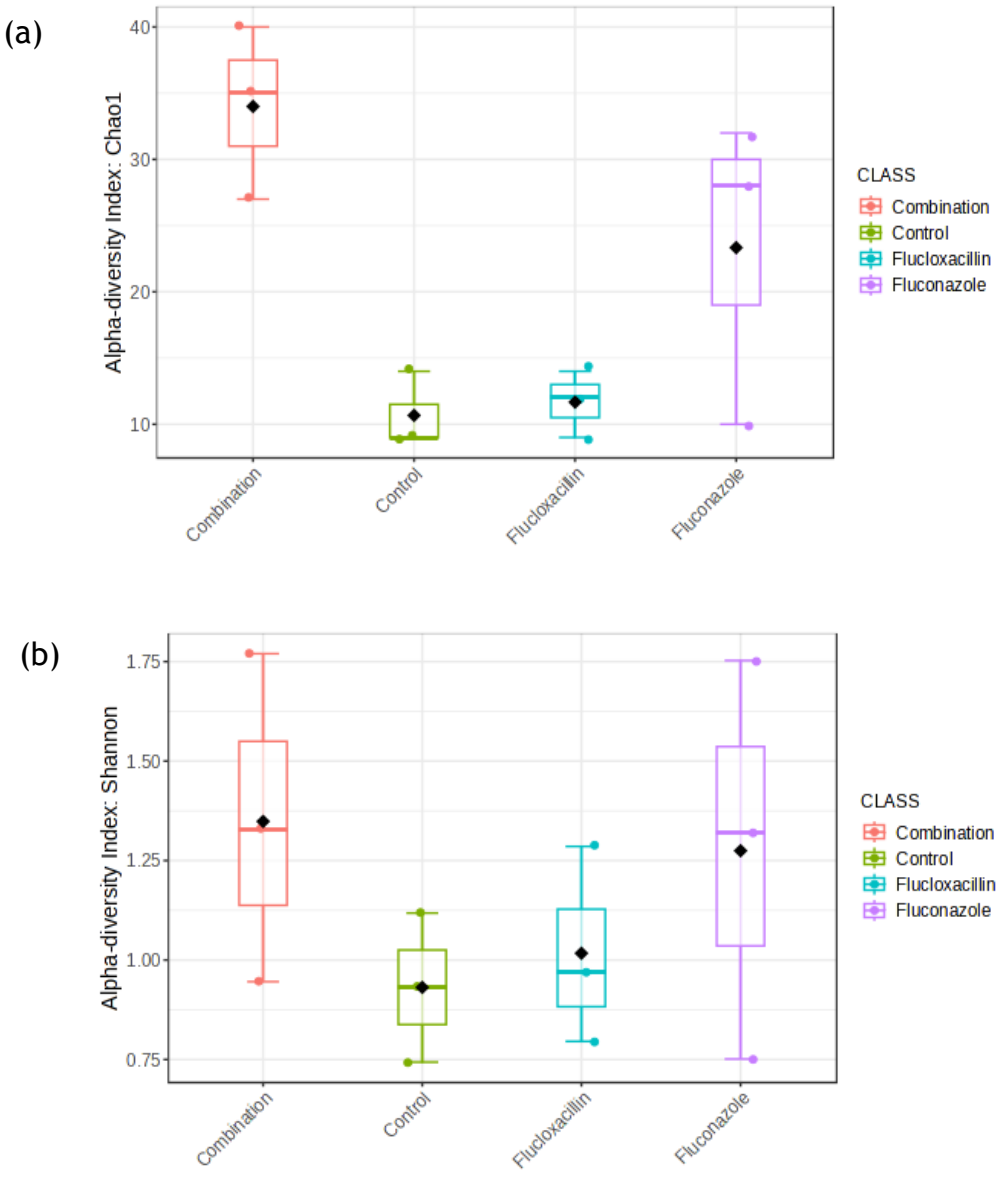


Figure 6.13 Alpha - diversity of treated biofilms from visit 7. Box- plots show the bacterial diversity within the sample of control and treated biofilms as measured using Chao1 **(a)** and Shannon **(b)** diversity indices. No significant differences ($p > 0.05$) observed for both indices using Kruskal-Wallis with post-host Wilcoxon pairwise comparison with multi-testing adjustment based on Benjamini-Hochberg procedure.

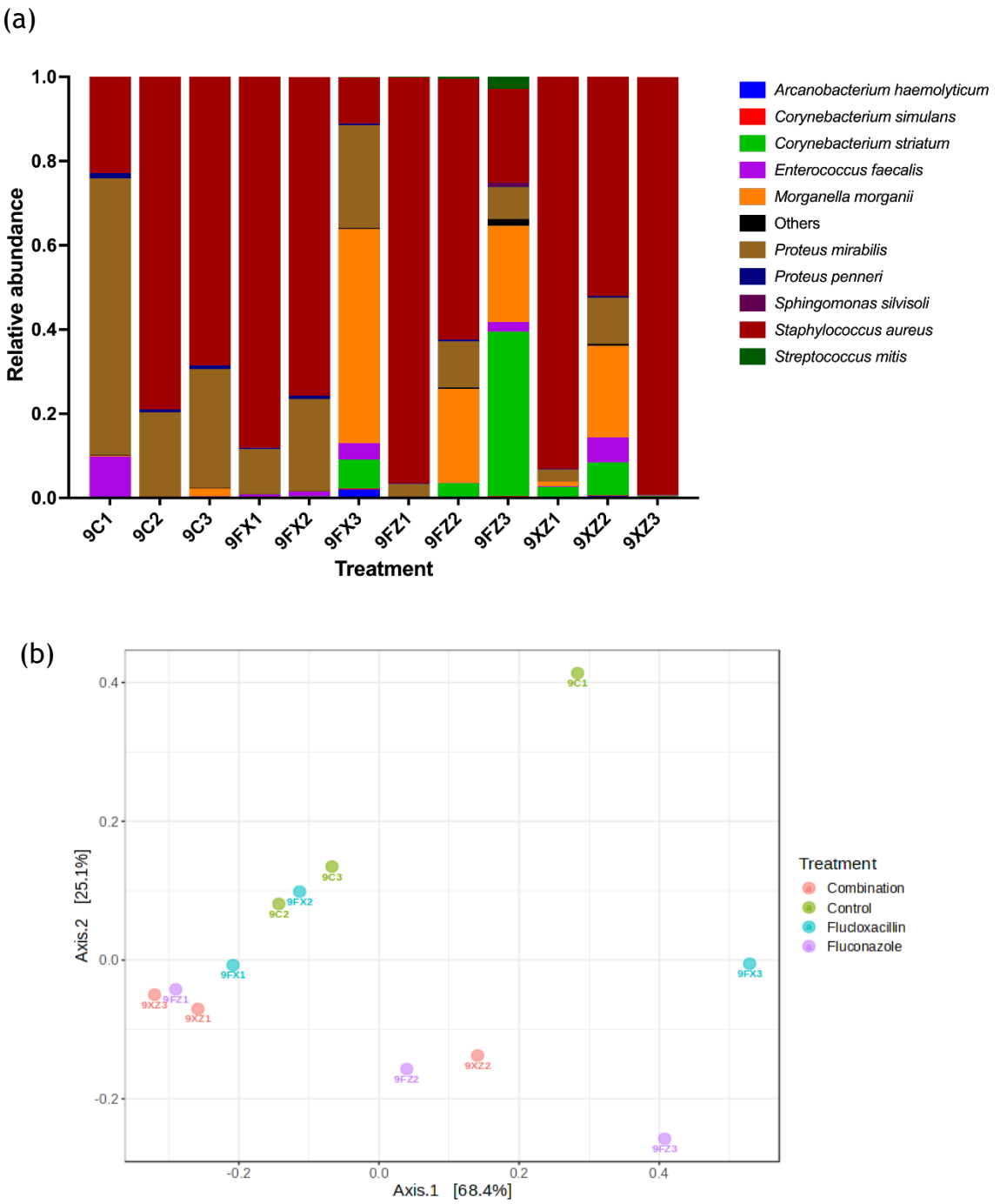


Figure 6.14 Microbiome composition and beta - diversity of treatment groups from visit 9. (a) Stacked bar plots demonstrate the changes in relative abundance of the top most abundance taxa between control (C), and treatments with Flucloxacillin (FX), Fluconazole (FZ), and combination of Flucloxacillin and Fluconazole (XZ). **(b)** PCoA plot with Bray – Curtis dissimilarity shows no significant difference (p >0.05) between treatment groups using PERMANOVA with multi-adjustment based on Benjamini – Hochberg procedure.

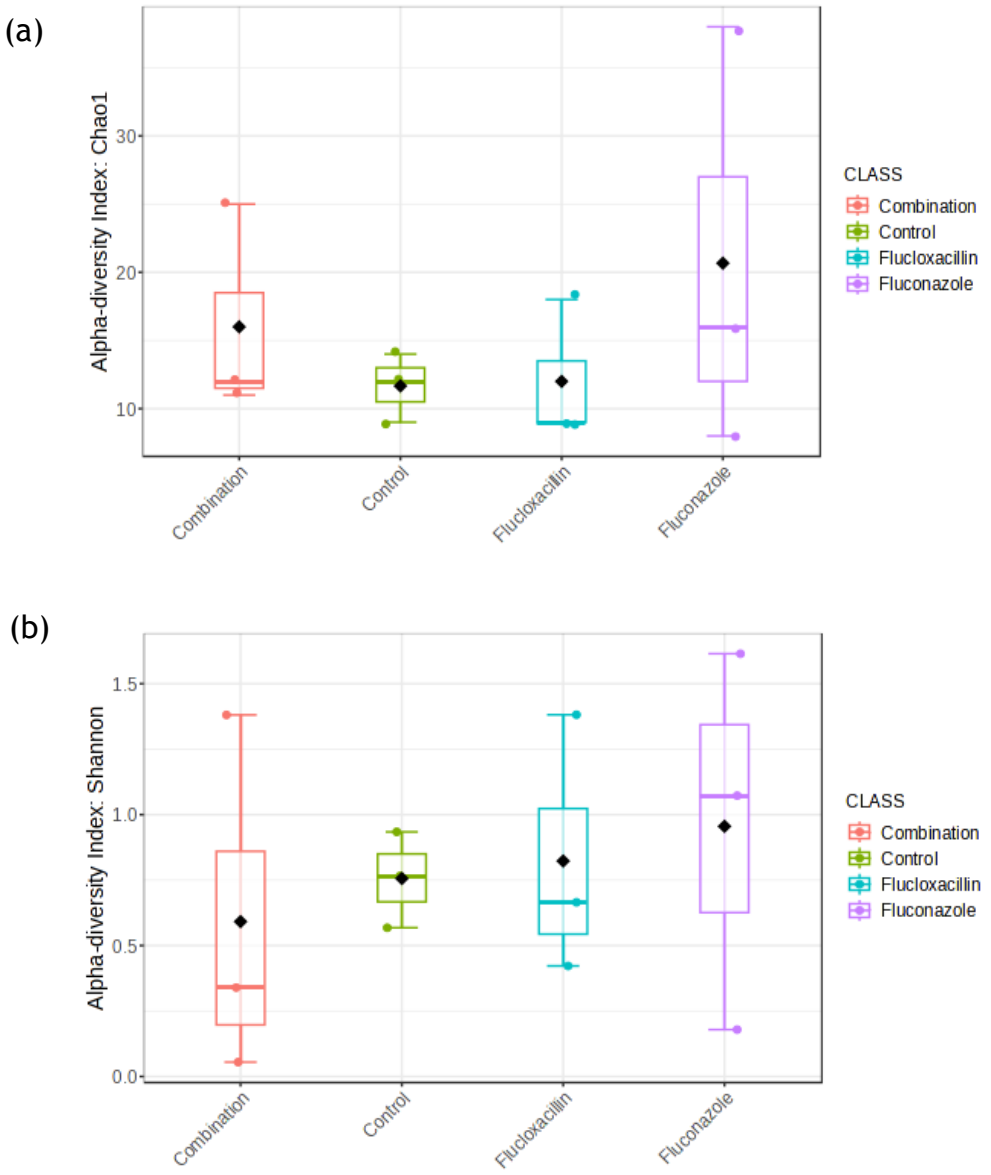


Figure 6.15 Alpha - diversity of treated biofilms from visit 9. Box- plots show the bacterial diversity within the sample of control and treated biofilms as measured using Chao1 **(a)** and Shannon **(b)** diversity indices. No significant differences ($p > 0.05$) were observed for both indices using Kruskal-Wallis with post-host Wilcoxon pairwise comparison with multi-testing adjustment based on Benjamini-Hochberg procedure.

6.5 Discussions

Prolonged antibiotic prescription is commonly seen amongst patients with severe diabetic foot ulcers (Soldevila-Boixader et al., 2023) with estimated spending of healthcare costs involving ulcer management and amputation ranging from £837-£962 million as reported in the NHS England (Kerr et al., 2019). The treatment may begin empirically with Flucloxacillin, Gentamicin, Clindamycin or in combination with several other agents and may get tailored accordingly to a more appropriate antibiotic regime following a microbiology culture and sensitivity results. Despite treatment, ulcer recurrence was not unusual after a complete healing with 40% reoccur within one year and was more difficult to manage than the initial wound (Guo et al., 2023). A similar rate of hospitalization was observed for patients who were empirically treated with antibiotics compared to culture-guided therapy (Schmidt et al., 2023). Some studies reported to have an increase in certain taxa following the antibiotics treatment although those taxa were different between studies (MacDonald et al., 2019; Price et al., 2009). Taxa such as *Pseudomonadaceae* were increased to an average of ~27-fold post-antibiotic treatment (Price et al., 2009). In contrast, Kalan and colleagues did not find that antibiotic therapy was causing changes to the microbiome in healed and unhealed wounds (L. R. Kalan et al., 2019). However, these observations were confounded by patient-specific characteristics including host immunity and PK/PD of the agents thus obscuring the measurement of the direct effect of the antibiotics. In the biofilm models presented in this chapter, the wound microbiome was found to be minimally impacted by the antimicrobials.

Whilst the microbiome of the wounds selected in this chapter were similar in the predominant taxa mainly *A. haemolyticum* and *P. melaninogenica*, these taxa were absent from the biofilms that were cultivated directly from the wounds in this study. These findings support the evidence that vast species of microbes found in DFU are difficult to culture although identified using molecular methods (Jneid et al., 2017). A slightly similar study of real-world biofilms that independent of predefined taxa was only able to capture two to six species of bacteria (Crowther et al., 2021). The attempt to incorporate the whole population resembling to the actual wound is indeed a very difficult and challenging task. So far, most of the *in*

vitro and *in vivo* wound biofilm models are based on a predefined composition of the microbes in regard to the species or inoculum concentrations (Ganesh et al., 2015; Short et al., 2023). To date, 11 species *in vitro* biofilms model is the largest number of species that have been incorporated in a defined wound model consisting of both bacteria (aerobes and anaerobes) and fungi (*C. albicans*) (J. L. Brown et al., 2022).

The four subsets of wound biofilms were composed of different dominant species. Overall pattern shows that each biofilm was predominated by at least two main species with all untreated biofilms consisting of *P. mirabilis*. Both biofilms from visits 2 and 4 consisted of *P. mirabilis* , with *E. faecalis* and *A. faecalis* as the second and first main taxa respectively. On the other hand, biofilms from visits 7 and 9 were slightly more variable but *S. aureus* appeared to be abundant alongside *P. mirabilis*. *S. aureus* and *E. faecalis* were commonly present in diabetic wounds as shown in the previous chapter and have been demonstrated in various biofilms models (Short et al., 2023). Interactions between *P. mirabilis* and *E. faecalis* have been commonly reported in catheter-related UTI (Learman et al., 2019). On the other hand, *A. faecalis* has been reported as a rare encounter in diabetic wounds but is often found in polymicrobial infections (C. Huang, 2020; Tena et al., 2015). Co-infection with *Morganella morganii*, a species under the tribe of *Proteeae* same as *P. mirabilis* has also been reported from the pus of diabetic foot with vascular ulcer(Tena et al., 2015). This result is somewhat interesting highlighting unconventional species in the wound biofilms model considering that the biofilms were formed without prior selection or defined concentration of the species during the experimental set-up. Moreover, this differs from our recent review of the mixed species biofilms models that show *S. aureus* and *P. aeruginosa* were frequently incorporated in the wound models (Short et al., 2023). Although *Enterobacteriaceae* group was among the most commonly identified in wound microbiome, there is still lacking evidence in studies that discuss their clinical importance in a chronic wound. Nevertheless, their capacity to form biofilms has been described in many studies (Kwiecinska-Piróg et al., 2014; Ramos-Vivas et al., 2019).

Similarly, the anaerobes including gram-positive anaerobic cocci (GPAC) and *Prevotella species* that were mostly present in the microbiome of the selected wound subsets also ceased to grow in our current model. This was disappointing

as in many cases, sampling and regrowing the anaerobes was hugely impacted by the sensitivity of the bacteria to the oxygen either during collection or sample processing. Despite success in cultivating various anaerobes in a defined wound model, Brown et al. have found suppression of *C. albicans* around ~50% under anaerobic conditions compared to when growing in 5% CO_2 (J. L. Brown et al., 2022). In addition, the CO_2 environment promotes biofilm formation by *C. albicans* (Pentland et al., 2021) . Here, *Candida* was seen to be lacking in hyphae and unevenly distributed in the biofilms when growing in CO_2 (Figure 6.5). As observed by Sherry et al., *C. albicans* may differ in morphology leading to variability in biofilm formation although these observations were limited to individual characterisation of the *Candida* isolates (Sherry et al., 2014). Thus, fulfilling the requirement of every single species under laboratory conditions was still a challenge thus far. In future investigations, it might be possible to incorporate the fastidious taxa by introducing them similar to a defined biofilms model with caution on the concentration of the inoculum used to mimic the actual burden. The biofilms model grown in this study was unable to incorporate all the main taxa that were present in each wound subset but instead was represented by relatively low abundance taxa.

A lot of attention has been focused on developing realistic wound biofilms model to mimic the actual wound condition. However, most components that have been studied only limited to growth conditions such as the media formulation and type of substrate for microbial growth (Sun et al., 2008). Moreover, most models have only been tested against a group of common wound pathogens primarily *S. aureus* and *P. aeruginosa* (Short et al., 2023), underrepresenting the actual microbial population and burden of the wound. Previous studies on defined models with either dual or multi-species have not been able to address a large number of taxa that are presence in the wounds as identified by sequencing (Jnana et al., 2020). The variability that emerges from using different models suggests that no one size fits all. Similarly, the contrasting pattern of biofilm microbiome between the subset of wounds observed in this study also suggest the variability that could emerge depending on the compatibility and interaction between the taxa when grown under laboratory conditions. Hence, this study suggests the possibility of microbiome sequencing analysis to be used in analysing chronic wound microbiomes in their native form for a comprehensive and precise composition of

the microbial population. Despite the aforementioned limitations, this study reveals a set of culturable and non-fastidious taxa in the biofilms model.

As far as antibiotics treatment is concerned, the microbial burden in all four biofilms was fairly stable despite the treatment with Flucloxacillin at 0.5 µg/mL. A much higher concentration of Flucloxacillin at 128 µg/mL alone is not effective against the triadic wound biofilms model consisting of *S. aureus*, *P. aeruginosa* and *C. albicans* (E. Townsend et al., 2017). In addition, this finding suggests that the concentration used during the treatment was insufficient to eliminate or inhibit bacterial growth in mature biofilms. However, this current study is not intended to investigate the effective concentrations to remove biofilms but rather to investigate the impact of systemic antibiotic concentration on the DFU microbiome. The use of antibiotic concentration similar to blister fluid or tissue concentration in this study should suggest the inadequacy of systemic therapy to eliminate the infection. It has been demonstrated that the Flucloxacillin concentration taken from the non-ischemic tissue of diabetic patients was more than 100 % less than the serum level concentration (Crowther et al., 2021). It seems possible that the wound would remain unhealed as biofilms may require aggressive treatment that cannot be achieved with systemic antibiotics alone. Further work is needed to identify or develop alternative drug delivery methods that can effectively release the desired concentration to eliminate the biofilms.

In addition, the microbiome of the biofilms from visits 2 and 4 were composed of predominantly gram-negative bacteria (*P. mirabilis* and *A. faecalis* respectively) which could also explain the inefficacy of the Flucloxacillin against these bacteria. However, biofilms from visits 7 and 9 were composed of *S. aureus* as the most abundant taxa and treatment with this antibiotic alone resulted in a slight compositional increase of other species in the biofilms. In other words, this indicates the susceptibility of the *S. aureus* to the Flucloxacillin but with limited anti-biofilm effect. In a much simpler biofilms model consisting of triadic species (*S. aureus*, *P. aeruginosa* and *C. albicans*), Townsend et al. (2017) observed a complementary replacement of other taxa in the biofilms when a single antibiotic that actively targeted a single species was used (E. Townsend et al., 2017). The elimination of one pathogen resulted in an overgrowth of others which may just prolong the chronicity of the wound as most bacteria can continuously grow and may become opportunistic in the absence of the dominant pathogenic taxa.

Therefore, physical intervention such as debridement has been shown to result in a substantial reduction of microbial burden in the wound, especially the anaerobes (L. R. Kalan et al., 2019). A study by Wolcott et al. on in vitro and in vivo biofilm models has also shown that debridement will remove the tolerance cells and allow the antibiotic to work against the exposed and newly grown bacterial cells within 24 hours post-procedure (R. D. Wolcott et al., 2010). This concept is interesting to be explored knowing that antibiotic alone has minimal activity on the microbiome in this present study.

In contrast to the antibiotics, there is much less information about the antifungal effect on the chronic wound microbiome. Three of the wounds (visits 2,4 and 7) were positive for *C. albicans* as identified from the enhanced culture in the previous chapter. Previous studies suggested a better prognosis of wound outcome when the patients were treated with antifungals (Raza & Anurshetru, 2017) and several studies have discussed a synergistic interaction between *C. albicans* and *S. aureus* by providing a scaffold for the bacteria to adhere during biofilm formation (Harriott & Noverr, 2009)(Kong et al., 2016). In this chapter, fluorescence imaging of the biofilm from visit 7 matches those observations from previous studies (Peters et al., 2010). However, the fungal load was inconsistent across the wound model to make a valid comparison. This may be due to the non-homogenous distribution of the fungi in the collected samples. Moreover, the fungal burden in the sample was also very low. Despite that, biofilms from visit 4 were more evenly distributed based on the ITS load compared to control, however were not impacted by any of the treatments.

On the other hand, *S. aureus* was significantly reduced in two of the biofilms from visit 7 with the combination treatment. It was uncertain to determine whether the reduction was due to the concomitant role of Fluconazole in eliminating *S. aureus* while targeting the fungi or due to the uneven composition of the biofilms. Nevertheless, this present study may suggest that a combination strategy with antifungals could have an additive advantage when *Candida* was present. However, this combination effect was only observed in biofilms that predominantly were gram-positive bacteria (*S. aureus*). Future work using an antibiotic that is active against gram-negative bacteria, especially the ones that were empirically used in DFU treatment such as Ciprofloxacin may reveal the effect of this antibiotic-antifungal combination drug strategy.

This present study provides the initial assessment of antimicrobial treatment on real-world biofilms from chronic wounds. The real-world modelling will prove useful in expanding our understanding on the impact of antimicrobials in this complex population in a controlled environment in vitro. This study was limited by the absence of anaerobes in the biofilms which might potentially shift the complexity of the chronic wound microbiome and the associated treatments. Notwithstanding the limitations, the study suggests that the right combination of antibiotics and antifungals at the appropriate concentration is crucial at eliminating the causative agents of infection. Considerably more work will need to be done to incorporate the absent microbes identified in the original swabs to elucidate a comprehensive impact of antimicrobial treatments while maintaining the population integrity of the wound.

Chapter highlights:

Antibiotic treatment has minimal impact on wound microbiome.

Appropriate selection of antibiotic and antifungal combination treatment may potentially enhance the elimination of causative microbes.

7 Final Discussion

7.1 Introduction

The present study was undertaken to improve understanding of the microbiome of stratified DFU according to different grades and stages and the implication of fungi on the dynamic of the microbiome through microbiological analysis of DFU swabs and real-world biofilm model. Certainly, prevalence data is what shapes the empirical guidance in treatment options for infected DFU. It was hypothesised that perhaps the microbial signature of more advanced DFU wounds was different from others, and these could be used to predict clinical outcome. Moreover, with a focus on *Candida* spp. and fungi within these DFUs, did these play a defined role. Despite the stratification of wound conditions, microbiomes are not entirely different between grades or stages, but instead revealed a unique profile in every wound that was analysed from multiple time points or visits. Moreover, the DFU microbiome is not solely composed of bacteria, but also a larger quantity of fungi. However, the fact that fungi are evident in chronic wounds, their presence has often been dismissed, and even more concerning when only bacteria have been reflected upon when anticipating the wound healing outcome and deciding on treatment. Currently, disintegration of the microbiome can be observed when bacteria and fungi have separately been studied. Although wide-ranging fungi have been identified from the literature, it is, however, the *Candida* genus that is predominantly identified in this present study. All of those strains were capable of forming biofilms, with some strains forming more robust biofilms than the others. This has been shown elsewhere and is critical factor in their pathogenic potential (Kean et al., 2018). Furthermore, this study represents one of the earliest attempts to utilise the undefined *ex vivo* wound biofilm model to model the antimicrobial effect from clinical use. An undefined collagen-based biofilm model has been shown elsewhere with the prior isolation of the microbiota from the wounds (Crowther et al., 2021).

7.2 Does one size fit all - implications for cross-sectional microbiome analysis?

Sequencing technology can capture an extensive number of microbial taxa compared to standard microbiology culture from DFU. Indeed, this is evidenced when an average of only 1.5 bacteria were identified from a single culture, but more than a hundred were detected with various sequencing technology platforms

(Jnana et al., 2020; Macdonald et al., 2021; Moon et al., 2021; K. Smith et al., 2016). This significant gap between the two methods demonstrates a large number of missing taxa unattainable by culture that can be revealed only by sequencing. Compared with other studies that utilized sequencing technology, the taxa prevalence is very similar in which *Corynebacterium*, *Staphylococcus* and *Streptococcus* are among the topmost common taxa identified (L. R. Kalan et al., 2019; Loesche et al., 2017; Sadeghpour Heravi et al., 2019). Generally, the microbiome of DFUs has remained unchanged, despite the fact that current guidelines for antibiotic coverage should work against these likely pathogens. However, DFU wounds can remain unhealed for a prolonged period, as observed in the subset of patients with multiple visits from Chapter 4. Moreover, the aforementioned organisms are the results of a snapshot of the microbiome from a single time point. In reality, the microbiome can be influenced by factors including antibiotics, behaviours and hygiene routines (Boxberger et al., 2021; Loesche et al., 2017; M. Zhang et al., 2015). A cross-section of the microbiome at any time point may capture a different population diversity. It is therefore indeed important to monitor the temporal changes that occur as the wound progresses, as has been described for some of the patients within the study.

From Chapter 4, the microbiome is found to be unique for every patient. In the subset of selected patients, none of the patients have the same microbiome composition at any time point. On the other hand, the same wound from the same patient can remain almost similar for a prolonged period. These differences will not be able to capture when only static time point data are being analysed independently. Furthermore, there is evidence that the wound is predominantly polymicrobial, and the concept of no single pathogen driving the DFU infection has been portrayed in a few studies (Dowd, Wolcott, et al., 2008; Loesche et al., 2017). Therefore, the dynamic of interaction between microbiota can only be obtained through longitudinal analysis of the wound.

As a skin break results in the creation of a new microbial niche in the form of a wound, there is no ‘normal’ microbiome state of the wound. Moreover, due to the open nature of the wound, constant exposure to microbes from various sources limits the identification of pathobionts, which were first described in a gut microbiome as a symbiont that normally cause disease in an altered host environment (Jochum & Stecher, 2020; Mazmanian et al., 2008).

7.3 How can wound microbiome offer more insight into the management of chronic DFU?

Currently, standard culture dictates the antibiotic treatment to be used against pathogenic bacteria in a clinical setting. Prior to the availability of antibiotic susceptibility results, empirical therapy should cover gram-positive cocci in mild infection with antibiotics such as Cloxacillin (Senneville et al., 2024). On the other hand, moderate to severe infection should include coverage of both gram-positive and gram-negative bacteria with antibiotics such as Amoxicillin-clavulanate (Senneville et al., 2024). Normally, no more than three organisms are reported to the clinicians, with each isolate accompanied by antibiotic susceptibility profiles. With ongoing treatment, succession or displacement of certain bacteria species may happen in response to the antibiotics (C. Liu et al., 2020). Standard culture will result in selective reporting of the potential organisms that thrive in the wound from a fraction of organisms that are capable of growing under limited laboratory conditions. With standard culture, the focus is to treat a single pathogen at a time. While this may eliminate the pathogen of interest, succession by other organisms especially multi-drug resistant organisms is a growing concern. In addition, one antibiotic after another will burden the patient's health such as a risk of renal toxicity, especially with prolonged antibiotic use. Therefore, chronic wound management demands a meticulous treatment plan before the start of the therapy.

Given a comprehensive microbial taxa from the microbiome data, early treatment can be tailored against a group of pathogens. As observed in Chapter 4, patients were repeatedly prescribed with antibiotics. The microbiome remains unchanged with unresolved infection in some patients despite treatment with recommended antibiotics from the standard culture. The targeted treatment that matches the standard culture may not have covered the potential pathogens revealed by microbiome data. Instead of successive prescriptions of antibiotics, a group of potential pathogens can be targeted simultaneously while minimising the impact on healthy microbiota. The coverage of antibiotics treatment based on potential infective pathogens recovered by the next-generation sequencing method has been estimated to lower the reinfection risk and better infection control (Kullar et al., 2023). The optimum use of antibiotics will prevent the emergence of multi-drug resistance organisms, thus promoting a good practice of antimicrobial

stewardship. The use of NGS has also been shown to support a reliable de-escalation of antibiotics treatment when accurate pathogens were identified (Zhou et al., 2021). In addition, antifungals should also be considered as part of wound management.

One of the factors that could potentially lead to inappropriate use of antibiotics is the uncertainty of the pathogens present in the wound (Lipsky et al., 2016). As shown in the microbiome chapter, a consortium of organisms was detected revealing more possible alternative treatments could be made. These treatment options may not necessarily target the most common pathogen such as *S. aureus* but also allow for multiple pathogens to be targeted simultaneously. With more taxa involved, the wound will be more complex to interpret, however, this should encourage more studies and clinical trials to be carried out on the use of microbiome data effectively in a clinical setting.

7.4 Does enhanced mycology culture improve fungal detection?

Culture may still be the best option to study fungi in clinical samples. According to the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC/MSGERC) consensus criteria, fungal isolation from culture can be the evidence to prove fungal disease (Donnelly et al., 2020). In contrast, a molecular method is still lacking standardisation to allow for an accurate diagnosis to be made. As highlighted in the literature review in Chapter 1, a vast number of fungal species has been identified in DFU, mostly from culture. Although fungal culture is time-consuming, as some fungi will take longer to grow, culture will allow species identification, and treatment can be adjusted according to the susceptibility profile of the fungal species isolated (Zhu et al., 2021).

Based on the data from standard microbiology culture, *Candida* is the predominant fungus isolated from the DFU (Chapter 2). Despite an enhanced mycology culture, species richness remains limited to mainly *Candida* genus. Unlike certain fungi, such as dermatophytes, *Candida* can already be visible as small colonies within 24 hours of incubation on standard microbiology medium such as blood agar (Barnes & Vale, 2005). In some cases, putative identification of *C. albicans* can be made

by the appearance of 'feet' surrounding the colony (Buschelman et al., 1999). Furthermore, the use of chromogenic agar will facilitate in distinguishing mixed species apart from providing a presumptive species identification based on the colour. It is apparent that the isolation of *Candida* should not be a challenge when standard microbiology media are used. However, caution about an overgrown by bacteria on standard microbiology media may necessitate the use of media supplemented with antibiotics, such as chloramphenicol to suppress or inhibit the bacteria growth (Hare, 2008). Moreover, fungi are usually present in lower abundance than bacteria. On human skin, fungal cells were found to be 1-2 $\log_{10}$ less abundant than the bacteria, and less than 1% in the gut (Gao et al., 2010; Huffnagle & Noverr, 2013). That being said, fungi are 100 times larger than bacteria representing a much higher biomass (Underhill & Iliev, 2014). Therefore, the use of supplemented media with antibiotics may enhance the growth and visibility of fungal colonies on culture media. While previous studies have isolated dermatophytes from DFU, these fungi were not observed in the present study. This group of fungi normally cause infection in the superficial layer of the skin and has rarely been reported to invade further (Wang et al., 2021).

With the evidence observed in this present study, enhanced culture may offer a limited advantage over standard microbiology culture. Yeast-like organisms such as *Candida* can be isolated together with bacteria in a standard bacteriological culture. However, suppression or inhibition of yeast growth on bacteriological media should be accounted for when polymicrobial growth is observed as this has been observed from previous studies (Sandven & Lassen, 1999). Moreover, when specific culture media such as SAB with chloramphenicol were used, fungal positivity did not show a significant difference compared to when using a standard bacteriology media.

Considering the evidence of fungi in diabetic wounds in non-healing wounds, treatment should not be limited to only antibiotics but should also consider the use of antifungals. A randomised control trial has shown a remarkable improvement in wound size when Fluconazole was combined with standard care in fungal-positive wounds (Chellan et al., 2012). The wounds that heal following antifungal treatments emphasize the importance of considering antifungals as part of standard care therapy when fungi are present (Heald et al., 2001). The presence of diverse bacteria and fungi in DFU wounds has increased the complexity of the

wound community. Therefore, neither bacteria nor fungi dominance should only be the focus when investigating chronic wound biofilms.

7.5 Is the real-world biofilm model a way to move forward?

The motivation for having a real-world biofilm model is to capture a consortium of microbes that are clinically isolated from infected DFU wounds. Based on the sequencing data presented in Chapter 3, the DFU wound is indeed polymicrobial. Moreover, taxa abundance does not exist in equal numbers reflecting mutualistic or antagonistic interactions within the microbial population. Therefore, modelling infection and assessing treatment is not as simple as creating a cocktail of microbes but also a community that resembles to the actual condition. The wound biofilms model as reviewed in Chapter 1 is very limited and selective towards the most common pathogenic species (Short et al., 2023). Although these organisms are high in virulence, they may not always co-exist in the same wound. From the standard culture in Chapter 2, *Pseudomonas spp*. is often found in mono-infection. Furthermore, the longitudinal analysis of individual patients in Chapter 4 reveals the absence of *P. aeruginosa* when *S. aureus* was present rather than the coexistence of the two species from the beginning. Despite being among the most common species in the wound, they do not necessarily co-exist together. Therefore, combining all the pathogenic species in the same model may not be representative, but could instead potentially deviate from the actual condition. Besides that, the selection of lab strains typically used in a defined biofilm model may underestimate the ranges of phenotypic differences from clinical strains (Fux et al., 2005). For instance, biofilms formed by different *Candida* species presented in Chapter 5 are distinct in biofilm biomass. Additionally, Sherry et al (2014) have reported that high virulence was associated with high biofilm former from different clinical strains of *C. albicans* (Sherry et al., 2014). Here, strain heterogeneity was shown to be an important determinant of clinical outcomes, above and beyond wounds.

Despite the attempt to simulate the microbial composition as close to the actual wound, both RWB and defined models are still restricted to the laboratory conditions. The impact is more profound in RWB when the viability of all microbes is uncertain prior to *in vitro* growth. Often, organisms that are easy to culture can

grow in biofilms while slow-growing or fastidious organisms may be undetectable. Therefore, reproducibility following RWB is difficult to predict.

With a defined polymicrobial biofilms model, factors including the number of species and inoculum density can be predetermined (Abusrewil et al., 2020). The models can recreate the microbial interaction to a certain limit. Currently, the 11-species wound biofilm model consisting of 10 bacteria and *C. albicans* is perhaps the most diverse biofilms wound model available (J. L. Brown et al., 2022). Despite the increased complexity of the model, strains that were used are from samples of various origins for example blood, lymph node and ovarian abscess (J. L. Brown et al., 2022). One of the drawbacks when combining different strains of different samples is the pathogenicity or the virulence trait of the strains might not be captured. Instead of replicating the wound, the model may result in artificial interactions that deviate from the actual wound.

7.6 Future work

In this thesis, works that have been done involve characterising the DFU microbial population including bacteria and fungi and the impact of systemic antibiotics on the dynamic of wound microbiome using a real-world biofilms model. During the longitudinal analysis of the subset of patients' microbiomes, the demonstration of the coexistence of fungi alongside bacteria for a prolonged period requires immediate attention. The current treatment management to tackle fungi should be investigated as antibiotics are ineffective against fungal growth. Moreover, polysaccharide mainly glucans produced by *C. albicans* serve as a protective barrier for *S. aureus* against antibiotics such as Vancomycin, rendering the treatment ineffective (Kong et al., 2016). As fungi and bacteria can form polymicrobial biofilms, assessing the antifungal treatment in combination with antibiotics when fungi are present is important. The real-world biofilms might serve as the foundation of a precision and individualised biofilms model to assess the impact of treatment in patients with unhealed wounds. In the real-world model presented herein, treatment does not include the critical step in managing biofilms in DFU, which is debridement. Biofilm-based wound management involves debridement as the initial step before antimicrobial treatment and incorporating this step would enhance the understanding of the optimum capacity of current treatment protocol against wound biofilms.

7.7 Concluding remarks

Taken together, the works presented in this study have highlighted the importance of interkingdom interaction between bacteria and fungi in a chronic diabetic wound. Both bacteria and fungi are essential components that drive the chronicity of the wound. Current antimicrobial therapy has only focused on pathogenic bacteria with guidelines that mainly include antibiotic agents. Antimicrobial therapy needs to cover bacteria and fungi when present simultaneously. The use of systemic antibiotics has minimal impact on effectively removing polymicrobial biofilms as demonstrated in real-world biofilm model. Insufficient antimicrobial concentration in the tissue fails to eradicate a consortium of microbes in the biofilm effectively. This study lays the foundation for future work in developing novel antibiofilm strategies that target not only bacteria but also fungi.

Key Findings

A graphical illustration of the important findings from this study is presented in Figure 7.1.

- DFU wound is polymicrobial which contains bacteria and fungi.
- As observed from Nanopore sequencing, DFU wound microbiomes are more diverse and patient specific.
- Candida is the most predominant fungi isolated from the DFU, with *C. parapsilosis* being the most common.
- Antibiotic treatment has minimal impact on real-world biofilms formed from clinical samples.

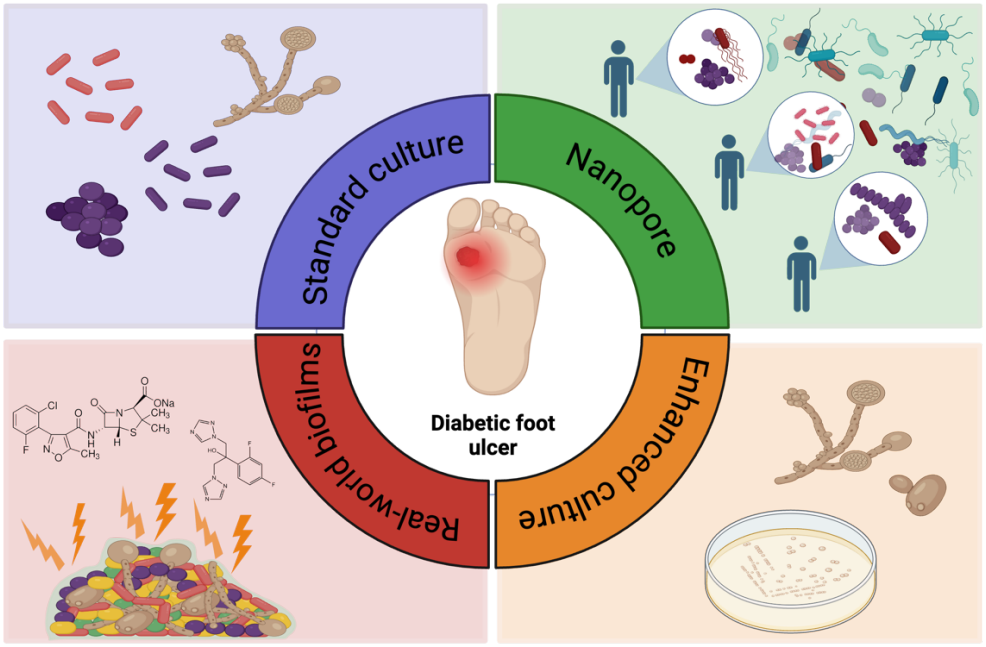


Figure 7.1 Key findings of this PhD study. Data from standard culture demonstrates a limited number of bacteria and fungi can be identified. Further application of the Oxford Nanopore sequencing platform has proven a more diverse microbiome of DFU. Moreover, the wound microbiomes are patient specific. Besides bacteria, *Candida* is the most common fungi isolated from the wounds. Antimicrobial treatments have minimum impact on the biofilms formed. Created with BioRender.com

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