The Oro-Nasal Cavity as an Endogenous Staphylococcal Reservoir for Diabetic Foot Ulcer Infections Investigated by Whole Genome-Sequencing and Disinfection of Debrided Foot Ulcer Tissues using Electrochemically-Activated Hypochlorous Acid Solution

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Declaration

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Summary

Background and study purpose

Type II diabetes mellitus is highly prevalent worldwide (approximately 6.3-10.5% of the global population), and in the population of the Republic of Ireland (9.5%), and continues to increase in prevalence. Diabetic foot ulcers (DFUs) and DFU infections (DFUIs) are complex and costly co-morbidities, affecting between 15-25% of people with diabetes in their lifetimes, and often leading to lower exteremity amputations. *Staphylococcus aureus*, a bacterial species highly prevalent in the oro-nasal cavity, is the predominant cause of DFUIs. The oro-nasal prevalence of this *S. aureus* is higher in people with periodontal disease (PD), an oral condition that is twice as prevalent in people with diabetes. This study sought to investigate the role of the oro-nasal cavity as a microbial reservoir for DFUIs and to determine if the electrochemically activated (ECA) disinfectant solution anolyte has potential as a novel therapeutic agent for treating DFUIs.

Methods

Ethical approval for all aspects of this study was granted by the Tallaght University Hospital (TUH) and St. James's Hospital (SJH) Joint Research Ethics Committee (REC) in January, 2020. Debrided DFU tissues were collected from consenting participants undergoing routine debridment therapy at the Diabetes Day Centre, St. James' Hospital, Dublin. Collected tissue samples (N=51) were divided into two equal aliquots by wet weight and comparatively subjected to immersion in either sterile saline or 200 parts per million (ppm) anolyte for three minutes. The first 20 samples were treated in 1 ml volumes and the remaining 31 were comparatively treated in 10 ml volumes to determine if higher anolyte volumes further improved disinfection efficacy. Following immersion, the microbial bioburdens of anolyte-treated and saline-treated tissues were compared following culture on Columbia blood agar (CBA) under aerobic and anaerobic conditions and on the staphylococcal-selective chromogenic agar (Sa*Select*™) under aerobic conditions. Microbial counts were recorded in colony forming units per gram (CFU/g) of tissue.

To explore the oro-nasal cavity as a potential microbial reservoir for DFUIs, consenting participants with type II diabetes with (N=76) and without (N=76) DFUs, underwent periodontal and DFU (if present) examination and clinical sample collection including saline oral rinse, sampling of two periodontal pockets, and a nasal, finger, toe and, if present, an ulcer swab. Oral and ulcer examinations were undertaken by a calibrated dental hygienist and podiatrist, respectively. Participants also described their oral hygiene routines using a self-reported survey. Staphylococci were recovered and presumptively identified on Sa*Select*™, and subsequently definitively identified by Pastorex-plus Staph Plus (Bio-Rad) latex agglutination testing, species-specific PCR, 16S rRNA sequencing and Matrix Assisted Laser Desorption Ionization Time-of-Flight-Mass Spectrometry (MALDI-TOF-MS) analysis. A selection of *S. aureus* (N=371) and *Staphylococcus epidermidis* (N=337) isolates chosen as representatives of distinct anatomical sites from participants both with and without DFUs underwent whole genome multilocus sequence typing (wgMLST) to examine the population structure of isolates recovered and to determine if oro-nasal and ulcer isolates were closely-related (≤24 wgMLST allelic differences), suggestive of endogenous transmission between these anatomical sites in the same individual.

Results

Significant reductions (P<0.01) were observed in the average bacterial load of 1 ml anolyte-treated tissues following CBA aerobic (227.7-fold [saline: $1.40\times10^6 \pm 5.28\times10^5$ CFU/g, anolyte: $5.11\times10^4 \pm 3.54\times10^4$ CFU/g]), CBA anaerobic (74.7-fold [saline: $9.23\times10^5 \pm 3.03\times10^5$ CFU/g, anolyte: $2.49\times10^4 \pm 9.14\times10^3$ CFU/g]) and Sa*Select*™ agar aerobic cultures (1605.6 -fold [saline: $6.29\times10^5 \pm 2.38\times10^5$ CFU/g, anolyte: $2.42\times10^4 \pm 1.48\times10^4$ CFU/g]). Further highly significant (P<0.01) fold reductions in average bacterial load (CFU/g) were observed in 10 ml anolyte treated tissues following CBA aerobic (12,243.6-fold [saline: $8.78\times10^5 \pm 3.50\times10^5$ CFU/g, anolyte: $2.86\times10^4 \pm 2.11\times10^4$ CFU/g]), CBA anaerobic (2747.2-fold [saline: $9.14\times10^5 \pm 4.68\times10^5$ CFU/g, anolyte: $4.13\times10^4 \pm 3.43\times10^4$ CFU/g]) and Sa*Select*™ agar aerobic cultures (9656.9-fold [saline: $1.67\times10^6 \pm 6.79\times10^5$ CFU/g, anolyte: $1.98\times10^4 \pm 1.33\times10^4$ CFU/g]). *Staphylococcus aureus* was the predominant organism recovered from tissues (30/51 tissue samples, 58.8%), of which 50 isolates representative of the 30 patients underwent wgMLST. The predominant sequence types (STs) identified included ST5 (N=18), ST1 (N=9) and ST15 (N=6). No methiciliin-resistant *S. aureus* (MRSA) or Panton-Valentine Leukocidin toxin (PVL)-positive isolates were detected in debrided tissue. Highly related isolates were recovered from nine separate patients who attended the same clinic an average of four weeks apart (range 1-16 weeks) between 2020-2023, indicative of transmission. However, as participants attend the DFU clinic weekly and participated in the study only once, definitive epidemiological data to support

transmission indicators was not available. Interestingly, 42/51 tissue samples (82.4%) yielded $>10^5$ CFU/g from saline-immersed samples. This is the threshold of microbial bioburden used to define of infection of DFUs and impede wound healing. However, in 39/51 (76.4%) of the patients sampled, there were no DFU clinical signs of infection suggesting that the clinical indicators used are not sufficiently sensitive for DFUI diagnosis.

Staphylococcus aureus was significantly more prevalent across all anatomical sites in patients with DFUs (nares [NS] 36/76 [47.4%], oral cavity [OR] 35/76 [46.1%], periodontal pockets [PPs] 13/54 [24.1%], finger [F] 10/76 [13.2%], toe [T] 11/76 [14.5%], ulcer [U] 38/76 [51.3%]) than participants without DFUs (NS 26/76 [34.2%], OR 25/76 [32.9%], PPs 9/76 [11.8%], F 4/76 [5.3%], T 3/76 [3.9%]). *Staphylococcus aureus* was identified in both the oro-nasal cavity and DFUs of 26/76 (34.2%) patients. *Staphylococcus epidermidis* was the most prevalent species identified overall in both participant cohorts. This species was also more prevalent in patients with DFUs (NS 45/76 [59.2%], OR 56/76 [73.6%], PPs 34/76 [62.9%], F 30/76 [39.5%], T 19/76 [25.0%], U 10/76 [13.2%]) than in those without DFUs (NS 39/76 [51.3%], OR 36/76 [47.4%], PPs 27/76 [35.5%], F 17/76 [22.4%], T 16/76 [21.1%]). *Staphylococcus epidermidis* was identified in both the oro-nasal and DFUs of 10/76 (13.2%) patients.

In total, 371 *S. aureus* isolates from patients with (*N*=266) and without (*N*=105) DFUs underwent wgMLST. The predominant *spa* types identified were t127 (*N*=43), t363 (*N*=19) and t230 (*N*=18) from patients with DFUs, and t084 (*N*=22), t908 (*N*=21), and t349 (*N*=19) for participants without DFUs. The predominant STs identified in isolates from DFU patients were ST1 (*N*=46), ST30 (*N*=33) and ST15 (*N*=29), and participants without DFUs included ST45 (*N*=32) and ST199 (*N*=17), typical of methicillin-susceptible *S. aureus* populations within Irish hospitals. Only one DFU patient yielded MRSA. PVL-positive isolates (*N*=35) were recovered from multiple anatomical sites of nine DFU patients. Of the 26 patients from whom *S. aureus* was recovered from both oro-nasal and DFUs, 19/26 (73.1%) of these paired isolates were indistinguishable or very closely related (median average: two allelic differences), indicative of endogenous transmission between anatomical sites. The majority of the oro-nasal isolates that were indistinguishable or very closely related to DFU isolates from the same patient were recovered from the nares (14/19 [73.6%]) indicative of a natural nasal niche for this species.

In total, 327 isolates of *S. epidermidis* were investigated by wgMLST from patients with (*N*=224) and without (*N*=103) DFUs. The predominant arginine catabolic mobile element (ACME) types identified were II (*N*=71), I (*N*=35) and V (*N*=11) from DFU patients, and III (*N*=61), II (*N*=43), I (*N*=35) from participants without DFUs. The *S. epidermidis* population was highly diverse. The predominant STs amongst DFU patients were ST5 (*N*=33), ST2 (*N*=21), ST87 (*N*=18) and ST35 (*N*=14), and participants without DFUs included ST35 (*N*=22), ST73 (*N*=10), ST218 (*N*=6) and ST153 (*N*=4). Interestingly, ST73 isolates were identified in 13 patients with (*N*=5) and without (*N*=8) DFUs. These were predominant in the oro-nasal cavity (19/23 [82.6%] from 13 patients) indicating possible enrichment of this ST in oro-nasal sites. Methicillin-resistant *S. epidermidis* isolates (*N*=12) were identified in three DFU patients and SCC*mec* elements (*N*=15) were identified in isolates from four DFU patients and one non-DFU patient. Closely related oro-nasal (oral [*N*=2], nasal [*N*=2]) and DFU isolates were identified in 4/10 (40.0%) distinct patients from whom *S. epidermidis* was recovered from both sites.

Conclusions

In conclusion, anolyte (200 ppm) is highly effective at reducing the microbial bioburden of debrided DFU tissues, particularly when treated with 10 ml volumes. These findings indicate that anolyte should be considered for a clinical intervention trial with active DFUs, rather than debrided DFU tissue. The prevalence of *S. aureus* and *S. epidermidis* is higher in DFU patients than patients without DFUs across all anatomical sites investigated. Either *S. aureus* or *S. epidermidis* were identified in both the oro-nasal cavity and DFUs of 36/76 (47.3%) patients with DFUs. Utilising WGS technology, highly related oro-nasal and DFU isolates were identified in 23/36 (63.9%) distinct patients with DFUs (*S. aureus* 19/23 [82.6%], *S. epidermidis* 4/23 [17.4%]), consolidating previous research that the oro-nasal cavity is an endogenous reservoir for DFUIs.

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Abbreviations

ACME	Arginine catabolic mobile element
AIDS	Acquired immunodeficiency syndrome
Ano	Anolyte
AR	Antimicrobial resistance
BLAST	Basic local alignment search tool
BLT	Bead-linked transposomes
BMI	Body mass index
BOP	Bleeding on probing
bp	Base pair
BSA	Bovine serum albumin
CA	California
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
CBA	Columbia blood agar
CC	Clonal complex
CFU	Colony forming unit
CFU/ml	Colony forming units per milliliter
CFU/g	Colony forming units per gram
cgMLST	Core-genome multilocus sequence typing
Co.	County
CoNS	Coagulase-negative staphylococci
CoPS	Coagulase-positive staphylococci
DUWL	Dental unit water line
DFU	Diabetic foot ulcer
DFUI	Diabetic foot ulcer infection
DNA	Deoxyribose nucleic acid
dNTPs	Deoxynucleotide triphosphate
ECA	Electrochemically activated solution
e.g.	*Exempli graita*; for example
et al.	*Et alia*; and others
EPM	Enhanced PCR mix
F	Forward/Finger
FAC	Free available chlorine
FEM	Flow-through electrolytic module

FL	Florida
Fold red	Fold reduction
g	Gravitational force
g	Gram
GD	Gestational diabetes
GP	General practitioner
h	Hours
Hba1c	Glycated haemoglobin concentration
HIV	Human immunodeficiency virus
HOCl	Hypochlorous acid
IBM	International Business Machines Corporation
ID	Identification
i.e.	*Id est*; that is
IEC	Immune evasion cluster
ILMN	Illumina
IWGDF	International Working Group on the Diabetic Foot
LEA	Lower extremity amputation
Ltd	Limited
Log red	Log reduction
M	Molar
MA	Massachusetts
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization-Time of Flight
mg	Milligram
$MgCl_2$	Magnesium chloride
MGE	Mobile genetic element
Min	Minute
ml	Millilitres
MLST	Multi locus sequence typing
mmol	Millimoles
mol	Moles
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
MS	Mass spectrometry
MSA	Manitol salt agar
MSSA	Methicillin-susceptible *Staphylococcus aureus*

MSSE	Methicillin-susceptible *Staphylococcus epidermidis*
MST	Minimum spanning tree
Mths	Months
mV	Milli-volts
N	Nares
N	Number
NA	Not applicable
NaOH	Sodium hydroxide
NB	Nutrient broth
NC	Negative culture
ND	Not determined
NGS	Next generation sequencing
nM	Nanomolar
NR	None recovered
NS	Not significant
NT	Non typable
NU	Non-ulcer
NY	New York
OC	Oral Cavity
OCL^-	Hypochlorite
OD	Optical Density
ONC	Oro-nasal cavity
OR	Oral rinse
ORP	Oxidation-reduction potential
P	Significant
P.	Proteus
PBP2a	Penicillin-binding protein 2a
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Periodontal disease
PFGE	Pulse-field gel electrophoresis
PP	Periodontal pocket
ppm	Parts per million
pH	Potential of hydrogen
pM	Picomolar

PVL	Pantone-Valentine leucocidin
ppm	Parts per million
R	Reverse
PBS	Phosphate buffered saline
REC	Research ethics committee
RHE	Reconstituted human epithelium
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Rotations per minute
RSB	Resuspension buffer
RT	Room temperature
s	Second
S.	*Staphylococcus*
SA	*S. aureus*
SAr	*Staphylococcus arlettae*
SC	*Staphylococcus capitis*
SCa	*Staphylococcus caprae*
SCC*mec*	Staphylococcal chromosome cassette *mec* element
SCr	*Staphylcoccus carnosus*
SE	*S. epidermidis*
SEq	*Staphylococcus equorum*
SEM	Standard error of the mean
SJH	St. James' Hospital
SH	*Staphylococcus haemolyticus*
SHo	*Staphylococcus hominis*
SK	*Staphylococcus kloosi*
SL	*Staphylococcus lugdunesis*
SLe	*Staphylcoccus lentus*
SP	*Staphylococcus pettenkoferi*
SPa	*Staphylococcus pasteuri*
SS	*Staphylococcus saprophyticus*
SSi	*Staphylococcus simulans*
SSu	*Staphylococcus sciuri*
SU	*Staphylococcus ureilyticus*
SW	*Staphylococcus warnerii*

SMRT	Single molecule real time
SNV	Single nucleotide variation
SNP	Single nucleotide polymorphism
spa	Staphylococcal protein A
SPB	Sample purification beads
spp	Species
SPSS	Statistical package for the social sciences
ST	Sequence type
T	Toe
TB1	Tagmentation buffer 1
TBE	Tris-borate/EDTA buffer
TE	Tris/EDTA
TGS	Third generation sequencing
™	Trademark
Tris	Tris (hydroxymethyl) aminoethane
TSA	Trypticase soy agar
TSB	Tagmentation stop buffer
TWB	Tagmentation wash buffer
U	Ulcer
US	United States
USA	Unites States of America
UK	United Kingdom
v3	Version 3
VF	Virulence factor
VRE	Vancomycin-resistant enterococci
v/v	Volume/volume
WHO	World Health Organisation
wgMLST	Whole genome multilocus sequence typing
WGS	Whole genome sequencing
µg	Microgram
µl	Microlitre
µM	Micrometre
-	Negative
%	Percentage
°C	Degrees Celsius

+	Positive
<	Less than
>	Greater than
~	Tilde; approximately
≤	Less than or equal to
≥	Greater than or equal to
× g	Earth's gravitational acceleration

Publications

Some of the original work presented in this thesis has been published in an international peer-review journal, as listed below. Offprints of the publication are included at the end of the thesis.

- **Grealy L., Wilson P., Gillen C., Duffy É., Healy M-L., Daly B., Polyzois I., Van Harten M., Dougall A., Brennan G. I., Coleman D. C., McManus B. A.** (2023). Immersion of debrided diabetic foot ulcer tissue in electrochemically generated pH neutral hypochlorous acid significantly reduces the microbial bioburden: whole-genome sequencing of *Staphylococcus aureus*, the most prevalent species recovered. *J. Hosp. Infect* 138:42-51 https://doi.org/10.1016/j.jhin.2023.06.006

Chapter 1

General Introduction

Introduction

1.1. Diabetes Mellitus

Diabetes mellitus is a chronic autoimmune condition of the beta cells of the pancreas that is characterised by insulin deficiency. Insulin facilitates blood sugar uptake into human cells and cellular metabolism. Insulin deficiency results in hyperglycaemia which leads to many widespread complications. Approximately 537 million individuals worldwide have diabetes. The estimated total number of people living with diabetes in Ireland was 308,948 in 2022 (1). Leahy *et al.* reported that the total prevalence of type II diabetes in the Republic of Ireland was potentially even 9.5% in 2015, with approximately 10% of cases undiagnosed. Diabetes is more prevalent in men than women and increases with advancing age (2).

There are two main categories of diabetes including type I diabetes and type II diabetes, as well as gestational diabetes and many subcategories all of which have different risk factors but have overlapping symptoms. Gestational diabetes (GD) generally subsides after pregnancy, however, women who have had GD are at risk of developing type II diabetes later in life (3). Ninety percent of patients diagnosed with diabetes have type II diabetes, a condition which is characterised by the inability of the body to produce insulin at sufficient levels. People with diabetes are at increased risk of multiple other complications such as periodontal disease (PD), due to increased salivary glucose levels (4), as well as urinary tract infections, high blood pressure, fatigue and neuropathy. Chronic hyperglycaemia is associated with long-term tissue damage and organ failure, involving the heart, kidneys, blood vessels and eyes. Neuropathy of the lower extremities often results in the development of diabetic foot ulcers (DFUs), which may hamper mobility and quality of life considerably (5).

1.2. Periodontal Disease

On a global scale, oral diseases are among the most common disorders among the general population, and oral health has been identified by the World Health Organisation as a major public health issue [5]. Periodontal disease (PD) is a common oral condition, manifestations of which accumulate with increasing age of the individual. Periodontal disease affects the dentition supporting structures which hold the teeth *in situ*. Poor oral hygiene leads to a build-up of microorganisms and dental plaque within gingival crevices, resulting in inflammation and ultimately leading to host immune-destruction of the periodontium (Fig. 1.1). Severe PD has been listed as the sixth most common condition globally, affecting near 734 million individuals [6].

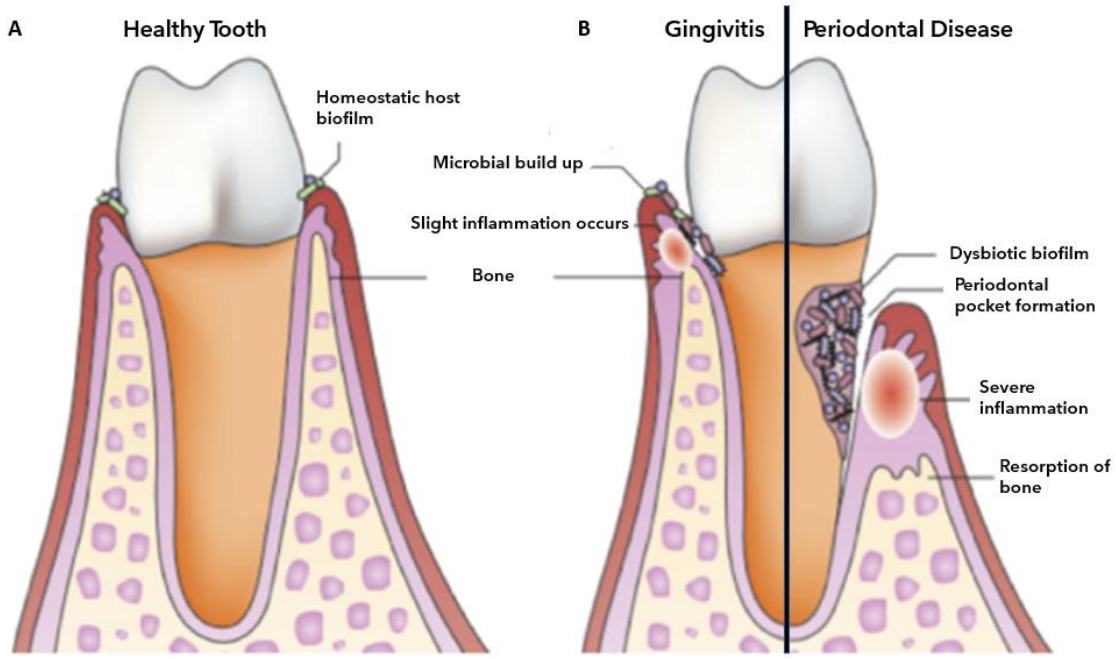


Figure 1.1. Schematic diagram showing the progression of healthy gums to inflamed gums with gingivitis, with unchecked gingivitis eventually progressing to periodontal disease. In tooth A, a healthy periodontium is shown with homeostatic biofilm formation within the gingival margin between the tooth and gums, which does not illicit an immune response from the host. In tooth B, gingivitis resulting from increased bacterial accumulation and host inflammation increases the size of the gingival margin. Unchecked gingivitis leads to the formation of periodontal pockets resulting from increased inflammation and other immune processes with the subsequent development of periodontal disease. Figure adapted from Hajishengallis *et al.* 2014 (9).

The periodontium is the region of oral tissues supporting each tooth and is composed of the gingival crevice, periodontal ligament, root cementum and alveolar bone. Periodontal disease is usually preceded by gingivitis, a more common, less invasive, and reversible form of PD whereby the gingival tissues become infected and inflamed due to bacterial accumulation. Visible dental plaque is generally evident on teeth 24 hours after undertaking oral hygiene and gingivitis develops following several days without oral hygiene. Unchecked gingivitis causes expansion of the gingival crevice, swelling and bleeding (6). If left unchecked, gingival detachment from the tooth results leading to the formation of a periodontal pocket in the periodontium, that permits the accumulation of dental plaque deep in the periodontal tissue. Dental plaque biofilms within the periodontium can prove resistant to host immune responses, blocking antibodies and immune cells from effectively targeting plaque bacteria. Ultimately the host immune response provides the most devastating destruction of the anchoring tissue leading to loss of supportive tissue, periodontal ligaments,

and reabsorption of the alveolar bone around the tooth (7). Predisposing risk factors for the development of PD following the onset of gingivitis include immunosuppression, smoking and poor oral hygiene. It is estimated that approximately 50-90% of adults in general, worldwide, have gingivitis, and 80% of adults will similarly have at least one tooth affected by PD during their lifetime (8).

It is well established that individuals with diabetes are more likely to develop PD than those without, and diabetes can play a major role in both initiation and progression of the disease (10). Studies on representative populations of patients have shown that diabetes can increase the risk of developing severe PD three-fold (11). Gingival crevicular fluids and saliva in patients with type II diabetes and PD have higher levels of inflammatory mediators including different cytokines compared to PD patients without type II diabetes (12). In adults, severe PD is one of the most significant predisposing factors for tooth loss, a major public health concern due to its effects on quality of life and significant effects on both oral and overall health (13). It has been reported that the likelihood of becoming edentulous, the permanent loss of all-natural teeth, is two times higher for patients with poorly controlled diabetes than for patients without diabetes (14, 15). Diabetes may enhance the risk of edentulism via direct oral complications associated with diabetes, such as inflammation of the gingiva, subsequent PD, and development of dental caries (15). A study undertaken by the US National Health and Nutritional Examination Survey reported that patients with diabetes were approximately 2.25 times more likely to be edentulous than individuals without diabetes (16). On average, one in five cases of edentulism in the USA is linked to diabetes (17).

1.3. Diabetic Foot Ulcers

The formation of DFUs is one of the most disastrous and prevalent complications of diabetes, which are common, complex and costly. Foot ulcers usually begin as a cut or abrasion of the skin and the gradual reduction in the thickness of the epithelium over time, leading to deeper erosion through the dermis into deeper tissues. As a result, diminishing blood flow via microvascular circulation obstructs both wound healing and phagocyte infiltration, thus impairing the host immune response (18). As well as the underlying diabetes-associated immunocompromisation, bacterial infection, ischaemia, progressive ulcer trauma and poor DFU management can further impair healing and promote the transformation of acute ulcers into chronic wounds. Diabetic foot ulcers are often categorised according to the Texas Wound classification system (Table 1.1) which grades DFUs according to wound depth, presence/absence of infection, and/or presence of ischaemia (19).

Table 1.1. University of Texas Wound Classification System for grading DFUs

	Grade [a]			
Stage [b]	0	1	2	3
A	Pre-ulcerative lesions, no skin break	Superficial wound No penetration [c]	Wound penetrating tendon or capsule	Wound penetrating bone or joint
B	With infection	With infection	With infection	With infection
C	With ischaemia	With ischaemia	With ischaemia	With ischaemia
D	With infection and ischaemia	With infection and ischaemia	With infection and ischaemia	With infection and ischaemia

Table adapted from Boulton *et al.* 2000 (20).

[a] Grade indicates the wound depth.

[b] Stage reports the presence of clinical infection (such as localised inflammation, purulent secretions and necrotic tissue presence) and ischaemia (diagnosed by poor reperfusion to the foot, or black or gangrenous toes) markers (21).

[c] No penetration indicates not all layers of the skin have been broken through by the wound/ulcer.

Ulceration of the lower extremities is commonly caused by a repetitive stress or injury over an area of the foot that is subject to high vertical or shear stress in patients with a condition known as peripheral neuropathy where nerves and nerve endings are damaged or diseased (22, 23). Ulceration very often results from the combination of injury and lack of sensation in the foot, enabling mild repetitive injuries or burns to go unnoticed and develop into an ulcer (24). Peripheral artery disease or ischaemia and neuropathy are both common chronic diabetes-associated complications [1], and, coupled with immune system dysfunction, are the main predisposing risk factors for DFU formation. A combination of ischaemia and neuropathy is reported to be the number one cause of non-traumatic lower extremity amputations (LEAs), increasing overall morbidity and mortality in patients [2]. At least half of all patients with diabetes develop neuropathy to varying extents during their lifetime [3]. Progressive nerve dysfunction and loss of lower extremity sensation often ultimately results in loss of balance, falls and a numb and insensate foot [4]. Intense or prolonged pressure from inappropriate footwear and contact-based on a locality already inflamed or structurally affected due to injury promotes DFU development [5]. Nearly 50% of patients with diabetes have peripheral arterial disease, defined as the partial or the

complete obstruction of the peripheral vessels resulting from atherosclerosis of the upper or lower extremity limbs, and patients with diabetes have a two-fold increased risk. Even minor injuries, particularly when complicated by infection, increase the demand for blood supply to the foot. Inadequate blood supply to the lower limbs can result in ulceration and impair wound healing thus an increased possibility of LEA [6].

According to a systematic review undertaken in 2016, the occurrence of DFUs in patients with diabetes ranges globally from 3-13% (25). Another study from 2017 reported that amputations were performed in 21% of patients who presented with DFUs (5). Patients with DFUs have an increased risk of mortality, which is estimated to be more than double that of patients with diabetes and without DFUs [7]. Moreover, DFU severity and types have been correlated with the highest rates of LEA and increased overall morbidity and mortality Morbach *et al.* determined that, in patients who had undergone major LEAs, cumulative mortalities at years one, three, five and ten were 15.4%, 33.1%, 45.8% and 70.4%, respectively [9].

Poor ulcer healing and recurrence leads to an increased risk of infection, resulting in the possibility of developing osteomyelitis, a severe complication associated with bone-deep DFUs stemming from soft tissue infections to infections of the bone and bone marrow, and subsequent foot amputation (26). Offloading weight (not bearing weight on the extremity or by fitting walking casts) while walking, or by not walking, along with other therapies such as surgical debridement and infection prevention measures (topical antiseptic treatment and oral or parenteral antibiotics), depending on the severity of the infection, can aid in the healing of a DFU. Effective healing of DFUs significantly decreases the risk of limb amputation or may avert it altogether (27).

1.3.1. Diabetic foot ulcer infections

All DFU infections (DFUIs) begin as superficial infections, predominantly caused by staphylococcal bacteria. As the infection progresses with an increased bacterial burden, DFUIs can progress into deeper, chronic polymicrobial infections. These are more difficult to treat due to the development of microbial biofilms in active ulcer sites, which are less responsive and often resistant to antibiotics used to treat such infections (28). Such infections result in a substantial increase in morbidity and mortality, more frequent healthcare visits and increases the risk of lower extremity amputation. Aerobic Gram-positive bacteria such as staphylococci and β-haemolytic streptococci are the predominant causes of DFUIs. Some patients that have undergone antibiotic therapy can be subsequently infected with Gram-negative bacteria. Patients who present clinically with foot ischaemia or gangrene in the

wound, may be infected with anaerobic bacteria such as clostridia (29). One study reported an abundance of anaerobic bacteria in new and recurring DFUs (30) The genera most commonly identified in new ulcers included *Peptoniphilus*, *Anaerococcus*, *Finegoldia*, *Porphyromonas*, and *Prevotella*. In recurring ulcers, the most common genera identified included *Finegoldia*, *Peptoniphilus*, *Anaerococcus*, *Porphyromonas*, with the addition of *Actinomyces* (30). Hospitalisation and surgical procedures, as well as failure to complete prescribed courses of antibiotics, can result in colonisation and infection by antibiotic resistant microorganisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant enterococci (VRE) (31).

1.4. Staphylococci

Staphylococci are a genus of Gram-positive bacteria characterised by their spherical shape and their formation of grape-like colony clusters. Most staphylococcal species associated with humans are both commensal organisms and opportunistic pathogens and cause a wide range of human ailments ranging from superficial skin infections to life-threatening bloodstream infections and pneumonia, as well as DFUIs. Research has identified *S. aureus* as the predominant pathogen in diabetic foot osteomyelitis, followed by *Staphylococcus epidermidis* (32). The isolation of *S. epidermidis* from bone biopsy samples and cutterage material from DFUs has led some researchers to propose that *S. epidermidis* be considered a nosocomial pathogen rather than a general skin commensal or contaminant in DFUIs (32, 33).

Staphylococcus aureus is a coagulase-positive (CoPS) species commonly associated with diseases ranging from superficial skin and soft tissue infections to more severe life-threatening illnesses such as infective endocarditis and necrotising pneumonia (34). *Staphylococcus epidermidis* is coagulase negative (CoNS) and is ubiquitous on skin and mucosal membranes. *Staphylococcus epidermidis* is typically a commensal organism but is often associated with infections caused by indwelling medical devices, including central venous catheters (35). Both *S. aureus* and *S. epidermidis* were also considered transient residents of the oral cavity, although previous research in this laboratory has reported these species are persistent in the oropharyngeal cavity (36). These species are also present in individuals suffering from oral infections, such as periodontal disease (37).

1.4.1. Staphylococcus aureus

Staphylococcus aureus is a commensal microorganism that typically resides on the skin, mucous membranes, intestines and especially within the nares of healthy individuals. Approximately 30% of the adult population are persistent nasal carriers of *S. aureus*,

however certain populations, including healthcare workers, tend to have higher rates of *S. aureus* nasal colonisation (up to 80%) (34). This species does not usually cause infection on healthy intact human skin, but if it gains access to the bloodstream or tissues, it can cause a wide range of potentially serious human infections, including impetigo, bacteraemia, infective endocarditis, osteomyelitis and prosthetic device infections (34, 38). *Staphylococcus aureus* colonisation generally precedes infection. Risk factors for infection include prolonged hospitalisation, immunosuppression, surgical procedures, the use of indwelling medical devices and chronic metabolic diseases (39). *Staphylococcus aureus* also can develop or acquire resistance to a wide variety of antibiotics classes including beta-lactams via horizontal gene transfer of mobile genetic elements (MGEs). Infections caused by MRSA generally result in higher mortality rates than methicillin-susceptible *S. aureus* (MSSA) isolates (40). The persistence of *S. aureus*-based DFUIs is also enhanced by staphylococcal virulence factors secreted by *S. aureus* (e.g. proteases, collagenase and lipases), which help to degrade the host tissue making it a more favourable environment for *S. aureus* to grow and colonise (41). Some strains of *S. aureus* can produce one or more additional exoproteins for immune evasion and colonisation (42). These exoproteins include toxic shock syndrome toxin-1 (TSST-1), and staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH, and SEI), which have dynamic effects on host immune cells, including inhibiting immune responses to *S. aureus* (42). One study reported up to 50% of lab rabbits hyperimmunised with TSST-1 failed to produce antibodies against the toxin, despite humoral responses remaining intact against other antigens (43).

1.4.2. Staphylococcus epidermidis

Staphylococcus epidermidis is the most prevalent coagulase-negative staphylococcal species on human skin and other epithelial surfaces (44). Although closely related to *S. aureus*, *S. epidermidis* is significantly less pathogenic. *Staphylococcus epidermidis* colonises the epithelium and mucous membranes and represents a major part of the human microflora (45), however, this species is often associated with nosocomial infections as an opportunistic pathogen in individuals with risk factors for infection. Very often, *S. epidermidis* becomes the predominant infective agent in immunocompromised patients, such as patients who inject drugs, those with AIDS, patients with diabetes and premature newborns (46). The ubiquitous presence of *S. epidermidis* on the skin permits the rapid colonisation of implanted prosthetic devices such as venous lines and urinary catheters. Unlike *S. aureus*, which has the ability to express a wide-range of virulence factors including extracellular enzymes and toxins that facilitate invasive infections in healthy hosts, *S. epidermidis* has relatively few

virulence factors in comparison and is normally unable to cause infection in an otherwise healthy patient (47). However, *S. epidermidis* constitutes a genetic reservoir for a diverse array of mobile genetic elements which can be transferred to other bacterial species, including *S. aureus*. Staphylococcal chromosome cassette elements (SCCs) harbouring the methicillin resistance-encoding gene *mecA* and associated genes are the most well characterised mobile genetic elements in staphylococci (48). There are at least 15 major SCC*mec* types (I-XV) (49), and several sub-variants that have been described in MRSA (50, 51) but many more types and subtypes have been described in *S. epidermidis*. These SCC*mec* elements integrate into the chromosomal *orfX* locus and often also contain virulence genes and other antimicrobial resistance genes (52).

Many isolates of *S. epidermidis* also harbour the arginine catabolic mobile element (ACME) composed of varying combinations of the *arc*, *opp3* and *kdp* operons, which facilitates survival in adverse environments and enhance its ability to colonise human skin and mucosal surfaces, allowing for its survival as a commensal on these surfaces (52, 53). Some of these ACME types (Type I; harbouring the *arcA* and *opp3* only, in the MRSA clone USA300, Type II; harbouring the *arcA* operon only) have also been described in *S. aureus* (54, 55).

1.5. Identification of staphylococcal species

A variety of selective chromogenic media have been developed to reduce the time required for the identification of specific bacterial species based on distinct colony morphology or colour (56). Sa*Select*™ is a good example of a selective chromogenic medium used for the presumptive identification of *S. aureus* and *S. epidermidis*; *S. aureus* generally yields pink/red colonies on Sa*Select*™, whereas *S. epidermidis* colonies are light pink/ white in colour (Fig. 1.2).

Other species of staphylococci can be distinguished using Sa*Select*™ such as *S. sciuri* and *S. lugdunensis* (yellow colonies), *S. intermidius* (grey/purple colonies), and other coagulase-negative staphylococci such as *S. haemolyticus* (white colonies). The use of chromogenic media facilitates the detection of multiple staphylococcal species which may be present in a clinical sample that may not be readily discerned using non-chromogenic media such as mannitol salt agar. Certain species of staphylococci can ferment the mannitol in this agar, such as *S. aureus*, then an acidic biproduct will be produced which will cause a phenol red pH marker in the agar to turn yellow (57).

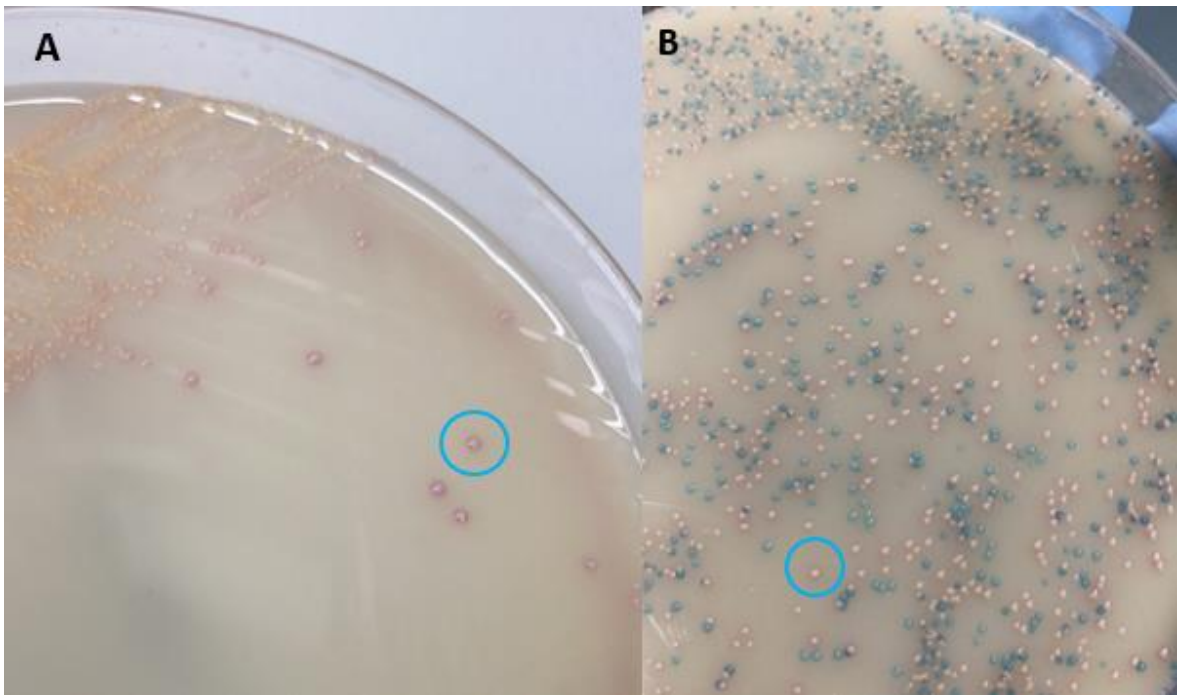


Figure 1.2. Photographs showing the appearance of staphylococci frequently recovered from clinical samples on chromogenic SaSelect™ agar after 24 h incubation. The blue circle in panel A indicates a presumptive *S. aureus* colony based on its red colour. The blue circle in panel B indicates a presumptive *S. epidermidis* colony based on its pale pink colour. Panel B also highlights the variety of staphylococcal colony colours that can be recovered on Sa*Select*™ following 24 – 48 h incubation such as *S. saprophyticus* (blue/green colonies) and *S. haemolyticus* or *S. warneri* (white colonies).

1.6. Molecular Typing Methods

Epidemiological investigations of microorganisms are commonly based on molecular typing approaches that investigate aspects of DNA in order to determine the genetic relatedness between isolates. Historically, phenotypic tests such as susceptibility to antibiotics were used to type isolates. These were then further investigated by traditional molecular typing methods based on enzymatically digested DNA such as pulse field gel electrophoresis (PFGE), comparing the size of chromosomal fragments generated by digestion with restriction endonucleases (58), and DNA fingerprinting methods including PCR, targeting specific DNA segments repeated at random in the bacterial genome (59). Although PFGE is a reliable typing method, it and DNA fingerprinting are both time-consuming and cumbersome, and in the 1990s, the application of exact DNA sequence determination and comparison led to the development of molecular typing methods based on nucleotide sequences directly. For example, since the large-scale replacement of PFGE, differentiation of *S. aureus* isolates has been based on variations in seven core loci using multilocus sequence typing (MLST), variations within the staphylococcal protein A (*spa*) gene

sequence using *spa*-typing PCR methods, characterisation of the SCC*mec* element and by elucidating the antimicrobial and virulence gene profiles of isolates using high throughput DNA microarray analysis (60, 61). Over the last two decades, earlier molecular typing methods such as these have been replaced by whole genome sequencing, which combines a lot of the earlier typing methods whilst also enabling vastly higher levels of isolate discrimination and comparison. Successful typing systems are high throughput and although expensive to use, generate large quantities of reliable and interpretable data (62).

1.6.1. Multilocus Sequence Typing

Multilocus sequencing typing (MLST) is a molecular typing technique used for differentiating isolates based on sequence differences in segments of selected housekeeping genes. This approach was developed to provide a reproducible method for the molecular characterisation of bacteria and was originally designed as a system to monitor global outbreaks of *Neisseria meningitides* (63). Minor sequence variations in housekeeping genes for each bacterial isolate investigated are assigned a specific allele number. The combination of allelic numbers for each of the loci per isolate are then defined as the bacterial sequence type (ST). The ST can be used as a specific marker to identify and differentiate isolates and establish the genetic relationships between related isolates using phylogenetic analysis tools, such as the minimum spanning tree (64). Since the advent of whole genome sequencing (WGS), species-specific MLST schemes such as core genome MLST (cgMLST) based on large numbers of conserved genome-wide genes have been developed for a range of bacterial species (65). These schemes are significantly more discriminatory than conventional MLST (64, 66).

Multilocus sequence typing can also be used in conjunction with *spa* typing of *S. aureus* strains, as well as identifying virulence and resistance genes utilising WGS to determine isolate relatedness. For isolates of *S. epidermidis,* whole genome sequencing can also provide data for ACME elements present within the genome of sequenced isolates similar to DNA microarray analysis. Coupled with MLST this can provide data to identify closely related strains. Sequencing techniques can also be used to visually display isolate relatedness for epidemiological investigations, as well as identifying genetic elements, and thus is the most useful molecular typing tool for such studies (67-69).

1.6.2. DNA microarray profiling

The DNA microarray platform has previously been utilised to screen large numbers of *S. aureus* isolates for detection of virulence and resistance determinants, as well as assigning *spa* types, sequence types and clonal complexes, and SCC*mec* types (70). Due to the close

genetic relationship between *S. aureus* and *S. epidermidis*, *S. aureus* DNA microarray technology can also be utilised to investigate the prevalence of virulence factor encoding genes in *S. epidermidis*, including ACME genes, antimicrobial resistance genes and SCC*mec* types (71), although it cannot be used for MLST analysis of *S. epidermidis* populations.

1.7. Whole Genome Sequencing

Sequencing technologies for determining the entire genomic sequence of microorganisms have undergone revolutionary advances during the last 20 years. These generational advances in sequencing technologies are now capable of determining the entire genomic sequence of a microorganism in a timely, relatively inexpensive approach. The various sequencing technologies currently available are referred to as First, Second and Third Generation sequencing, and each technology has its own advantages and disadvantages.

1.7.1. First Generation Sequencing

First generation sequencing involves two DNA sequencing techniques from the 1970s including Maxam-Gilbert Sequencing and Sanger-Sequencing, which were able to sequence several hundred base pairs at a time (69). Maxam-Gilbert sequencing is a method for DNA sequencing developed in 1977 and was a significant breakthrough, allowing for the direct sequencing of purified DNA without requiring previous *in vivo* cloning of the DNA and single strand DNA preparation steps (72). It involves a chemical degradation of the DNA where the DNA is radio-labelled and split into four sequence reactions. Sanger sequencing involves an enzymatic chain termination reaction and generates easily interpreted raw data (73). Sanger sequencing remains the only first-generation sequencing technology still routinely used for small scale DNA sequencing (74).

1.7.2. Second Generation Sequencing

Second generation sequencing can generate enormous quantities of sequencing data (15 gigabytes of data per sequencing run) at a relatively moderate cost, and thus has revolutionised the field of microbial genomics (69). These second, or next, generation sequencing technologies, including whole-genome sequencing (WGS) have begun to rapidly dominate modern-day DNA sequencing activities, generating large quantities of sequence data, e.g. the Illumina MiSeq platform can yield 25 million reads at a rapid rate. Among these available systems, the platforms provided by Illumina Ltd. predominate. These platforms utilise a sequencing by synthesis approach (75, 76) which involves DNA fragmentation, library preparation, parallel sequencing and subsequent bioinformatic analysis based on mutation annotation and interpretation of results (77) (Fig 1.3).

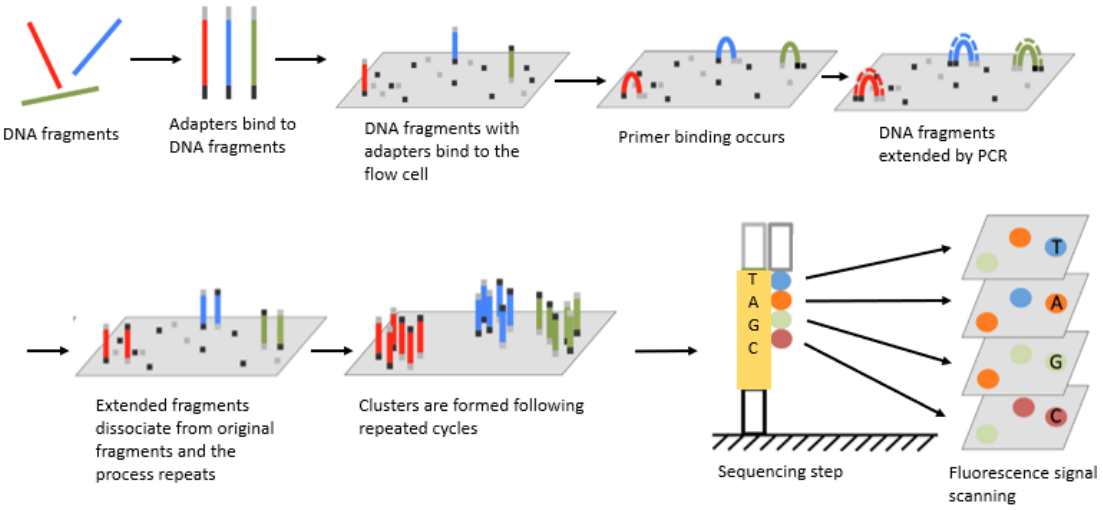


Figure 1.3. Schematic diagram showing the process of second-generation sequencing. DNA is fragmented and adaptors are annealed to the ends of the fragments. Adaptor-bound DNA fragments bind to specific primers on a primer loaded flow cell, and bridge PCR reactions amplify each bound fragment to produce a cluster of DNA fragments. During the sequencing cycle, a fluorophore attached nucleotide is attached to the growing DNA strands. A laser excites the fluorophores in the fragments being sequenced, and an optic scanner collects signals from the clusters of DNA fragments. The sequencing terminator is then removed, and the next sequencing cycle begins. Figure adapted from Lu *et al.*, 2016 (78).

The Illumina sequencing by synthesis approach involves several library preparation steps using extracted, high-quality DNA. This purified, high-quality DNA is fragmented either by mechanical or enzymatic shearing and unique adaptor sequences known as indexes are ligated onto each DNA fragment. These adaptor-bound DNA fragments are loaded into a reagent cartridge which is inserted into a high-throughput DNA sequencer, such as the Illumina MiSeq DNA sequencer. This reagent cartridge automatically incorporates the mix of reagents and DNA fragments onto the solid surface flow cell that is coated with both primers complementary to the adaptor sequences. These primers recognise the indexes attached to the DNA fragments. These bound end fragments then bind to the surface of the flow cell, and a DNA polymerase amplifies the fragments to produce several identical copies, called DNA clusters. The MiSeq platform uses a four-fluorophore system, and thus four different fluorescently-tagged nucleotides are added to the flow cell and are incorporated by a DNA polymerase into a new DNA strand, one nucleotide at a time. These are washed and cleaved, and the fluorescence step is repeated. The sequencer documents the colour changes after each nucleotide addition to construct the genetic sequences of the DNA

clusters. This results in a comprehensive analysis mechanism to study entire genomes and has been used to study multiple species of organisms, from bacteria to humans (64). The MiSeq platform is generally used in clinical diagnostic laboratories and research laboratories (79). Although second-generation sequencing platforms generate large quantities of sequencing data, there are several limitations to their usage. These sequencing technologies require sophisticated bioinformatics systems, fast data processing capabilities and need significant storage space for the large quantities of data generated. Other shortcomings from second-generation sequencing technologies include their inability to sequence large MGEs, such as plasmids, SCC*mec* elements and lysogenic prophages due to unreliable assembly of short read, repeat-containing DNA sequences.

1.7.3. Third Generation Sequencing

Third generation sequencing refers to new technologies that can produce substantially longer reads compared to NGS. Commercially available third-generation sequencing technologies include the Illumina Tru-seq synthetic long read technology, Pacific Biosciences (PacBio) Single Molecule Real Time (SMRT) sequencing and the Oxford Nanopore Technologies sequencing platform. These three technologies can produce long DNA reads between 5000-15,000 bp, with some reads exceeding 100,000 bp. This can be achieved using single molecule sequencing or clonal amplification of larger molecules (80). However, NGS is generally the primary choice of sequencing platform as the larger reads are more prone to error on TGS platforms (81). Over the last ten years, researchers have developed bioinformatic tools to generate hybrid sequence assemblies based on both NGS and TGS sequence data. Recently developed hybrid approaches can be used for assembling long (inaccurate) and short (accurate) reads, which has proven to be useful in generating high quality reads at low cost (82, 83).

1.8. Epidemiological typing of microbial pathogens based on WGS data

Next and third generation sequencing technologies require complex laboratory workflows and generate high throughput data that relies on bioinformatic processing, analysis and interpretation (84). The majority of WGS-based typing methods use NGS technologies, predominantly based on the Illumina platforms which the generated single-end reads are packaged into a single FASTQ file, paired end reads generate two FASTQ files (85). These FASTQ files contain all sequence read data per isolate. However, it is necessary to undergo several analysis steps to access the genomic data generated from the sequencing run. These analyses are often undertaken using online commercial software packages using graphical user interfaces, particularly in clinical laboratories.

1.8.1. cgMLST

The core genome consists of genes which are shared by much of a species. These genes have been subjected to much stronger purifying selection than more mobile elements of the genome, which are referred to as the accessory genome (86). Core genome MLST (cgMLST) is an extension of conventional MLST which greatly increases typing resolution without the requirement of a reference genome. The method involves gene-by-gene comparisons of each locus from the core genome to generate an allelic profile for each bacterial isolate (81). This makes cgMLST a useful tool in evaluating groups of isolates acquired over an extended period of time, for studying groups of isolates that are not as closely related at the population level. The current widely used *S. aureus* cgMLST scheme includes 1,861 loci, seven of which form the traditional MLST scheme (87).

1.8.2. wgMLST

Whole genome MLST (wgMLST) is a further extension of cgMLST that also includes loci from the accessory genome, which further enhances resolution. This tool is effective in investigating closely-related isolates in local settings and for both outbreaks and patient infection studies, such as multiple isolates recovered from the same patient (87, 88). Allelic variations in nucleotide sequences are defined into categorical characters from a set of thousands of gene loci. This process has also proven to be very useful in tracking bacterial pathogen outbreaks (89) and patient infection studies (67, 90).

1.8.3. SNV Analysis

The systemic study of single nucleotide variation (SNV) is one of the most useful approaches to delineate and differentiate cellular heterogeneity at the single cell level (91). One study used SNV analysis, for example, and identified an *E. coli* isolate in a wastewater treatment plant which exhibited marked clonality, showing less than 12 SNVs with clinical isolates from several countries (Southeast Asia, EU, and Oceania) (92). It has also been used to track nosocomial outbreaks MRSA between patients and environmental sites (93). This analysis involves the use of one reference genome to comparatively detect SNVs throughout the entire genome under investigation. This tool can be used based on cgMLST, wgMLST and WGS data, where WGS can reveal SNVs in microbial cellular populations (68).

The advent of WGS and development of bioinformatic comparative analyses such as cgMLST, wgMLST and SNV typing presents unique research opportunities, allowing investigators to directly compare isolates in a highly discriminatory and reliable way. This technology allows the detection of sequence variation with highly sensitive and reproducible approaches including globally based epidemiological and population studies, enabling

comparison between different species and within the same species. This highly discriminatory method may be highly useful in the identification of infection reservoirs which may be present in humans, animals and environmental sources, allowing for potential mechanisms of intervention to mitigate further infection.

1.9. The link between the oro-nasal cavity and DFUIs

Many of the bacterial pathogens that present the greatest burden to human infections are endogenous in nature and are frequently found as commensal microorganisms such as *S. aureus* (94). An association between microbial pathogens in the oro-nasal cavity and systemic infection has been well documented (95). It is becoming increasingly recognised that oral infections, such as PD may influence a number of systemic diseases such as cardiovascular disease, bacterial pneumonia and diabetes. These include (i) the metastatic spread of microorganisms from the colonisation/infection site in the oral cavity resulting from transient bacteraemia, (ii) metastatic injury from microbial toxins in the mouth and (iii) metastatic inflammation caused by the build-up of oral microorganisms (96). Advances in molecular typing and sequencing technologies have transiently-detected oral microorganisms within many organs in the body, including the heart, placenta, small intestines and the brain (97). The possibility of physical transmission of oral bacteria to other sites is also significant. This can result in superficial infections which can lead to deeper, chronic infections in open wounds, such as DFUIs. Dunyach-Remy *et al.* (2017) reported that nasal carriage of *S. aureus* was an endogenous reservoir for DFUIs utilising DNA microarray analysis to identify closely related isolates within the nares and DFUs of patients with DFUs (98). McManus *et al.* (2020) utilised WGS to identify both the oral cavity, periodontal pockets and nares as a potential endogenous reservoir by reporting highly-related *S. aureus* isolates in the oro-nasal cavity and DFUs of patients with type II diabetes and DFUs (99). The latter investigation was a pilot study, and required a larger population of both patients with and without DFUs to consolidate the study data (99). Therefore, the oro-nasal cavity may be a potential reservoir for severe systemic and chronic infections in patients with diabetes. The occurrence of such systemic and chronic infections is disproportionally higher in patients with diabetes than for individuals in the general population, which can be caused by the hyperglycaemic environment which favours immune dysfunction (100).

1.10. Electrochemically activated solutions

Electrochemically activated solution (ECA) technology was developed as a specialised discipline of electrochemistry by Professor Vitold Bakhir in the former Soviet Union in the

1970s (101). ECA solutions are generated by passing a dilute salt solution through an electrical field in a flow-through electrolytic module (FEM) and separating the reactive solutions generated. The activation process changes the state of the salt solution from a stable to a metastable state. This electrochemical activation generates two oppositely charged solutions both possessing different physical and chemical properties (102). The positively charged solution, termed anolyte, consists mainly of metastable hypochlorous acid and has a redox value of approximately 600 mV and other unstable mixed oxidants. These mixed oxidants are held together in a physically excited state. Hypochlorous acid is highly microbicidal and can penetrate and destroy microbial biofilm. The negatively charged antioxidant solution, termed catholyte, is composed primarily of sodium hydroxide and has detergent properties. Catholyte typically has a pH of 11 and a redox value of -600mV. These free radicals and active ion species are short-lived, with a half-life of approximately 48 h (103). Different kinds of ECAs have been developed following the advent of this technology and are produced by different types of generators with specific configurations. Older model ECA generators tended to produce acidic anolyte solution with inconsistent properties. The Trustwater Group (Clonmel, Ireland), Clarentis (Clarentis Technologies, FL USA) and Envirolyte (Envirolyte Industries International Ltd., Estonia), ECA generators have refined technology and can be configured to produce anolyte at a neutral pH, which is the anolyte used in this study. It differs greatly in comparison to the acidic anolyte produced previously which is highly corrosive (104).

Numerous studies over the past three decades have shown that output water from dental unit water lines (DUWLs) is heavily contaminated with microorganisms (105-110). These microorganisms can build up and form thick biofilm in DUWLs (102). Electrochemically-activated hypochlorous acid solutions have been shown to minimise contamination and biofilm formation in DUWLs. One study showed that the average bacterial bioburden in unprocessed mains water and anolyte-treated mains water from DUWLs was 88 colony forming units (cfu)/ml and <1 cfu/ml, respectively (102). It was shown, more recently, in another long-term (one year) study, that microbial contamination of clinical washbasin tap output water (both hot and cold) and associated taps can consistently be minimised by residual treatment of supply water with anolyte (111).

Biofilm present in hospital wastewater pipes and washbasin U-bends have also proven to be difficult to eradicate (112). U-bends are wet, nutrient-rich segments of the wastewater pipework used to prevent ingress of sewer gas from wastewater pipes into buildings, and are frequently coated in dense biofilm. Automated U-bend decontamination systems have been used to eradicate these biofilm-forming microorganisms from hospital U-

bends (113). These systems utilise a combination of catholyte followed by anolyte to control U-bend biofilms. Several studies have shown that water from ECA-treated U-bends saw significant reduction of micro-organisms in comparison to non ECA-treated U-bends (113-115). Electrochemically activated solutions have also been used as a disinfectant in Russia for over 30 years for the disinfection of drinking water, swimming pools, hospital water lines, for irrigating wounds and for destroying microbial biofilms (103, 104).

1.10.1. Electrochemically activated solutions in wound treatment

Chronic wounds are a significant burden to patients all over the world, and account for approximately $20 billion per annum in healthcare costs. Ulceration of the lower extremities is the leading international cause of amputation and infection of ulcers only exacerbates this issue. There are however a number of therapies used to combat DFUIs. The most commonly used treatment for DFUIs is debridement therapy, where infected tissue is 'cut out' from the wound, promoting tissue healing and growth, and systemic antibiotic therapy to control the growth and spread of harmful microorganisms (116). Topical antiseptic compounds have been sometimes used in preference to antibiotics to avoid the development of antibiotic resistance among microorganisms resident on or in infected tissue, and although infection may slow the healing process, overuse of topical antiseptics may also inhibit the healing of the host tissue (117). The use of antiseptics such as dilute acetic acid, acetyl alcohol, hydrogen peroxide and sodium hypochlorite has decreased due to adverse effects on fibroblasts required for epithelisation (116).

Super-oxidized solutions or ECAs could provide an alternative approach to the use of antiseptics for the cleaning and disinfection of infected wound tissue and surrounding skin such as DFUIs. Electrochemically activated hypochlorous acid solutions have been effective in the treatment of various wounds ranging from septic wounds, surgical wounds, chronic wounds and DFUIs (116, 118-120). These powerful solutions are able to destroy *Enterococcus faecalis* biofilm from the root canals of extracted teeth (121) as well as *S. aureus* and *Candida albicans* from dental prostheses (122). One study has shown that TR146 human keratinocyte monolayers and reconstituted human epithelium (RHE), pre-treated with bovine serum albumin (BSA) equivalent to protein layers in human saliva, exposed to anolyte displayed no cytotoxic effects. It was reported that 100 x 2.5 ppm did not display any detectable adverse effects on human keratinocytes (123). This indicated that residual anolyte at 2.5 ppm used to continuously decontaminate dental unit DUWLs would not have serious side effects on human oral tissue. It has been documented for over a decade that ECA solutions have the potential to treat diabetic foot ulcer infections, resulting in lesser healing

times and no adverse side effects (116). Dalla-Paola *et al.* (2006) reported that anolyte used to treat DFUIs had better treatment outcomes in comparison to topical antiseptic treatments, including the reduction of bacterial density within the tissue via the killing of resident bacteria, improved healing times and less local adverse effects (116). The findings from this study support the safety of anolyte in wound care. Antiseptics generally cause increased cytotoxicity in fibroblasts, but this is not the case for anolyte which has been shown not to induce cytotoxicity in fibroblast populations, thus increasing wound healing process and reducing overall chances of further infection, which may be hampered by conventional wound treatments (124).

1.11. Project Aims

Previous research from this department reported that the oral prevalence and abundance of staphylococci is significantly higher in patients with periodontal disease (48). Oral health is a commonly overlooked and under researched area in diabetes management. The increased risk for people with diabetes to develop PD and DFUIs and the association of staphylococcal species with both diseases suggests an urgent need for research investigating the role of the oro-nasal cavity as a microbial reservoir for DFU infection, as well as better understanding the role of oral health in diabetes management.

This research aims to investigate and assess the oral health of people with diabetes attending a Dublin-based acute diabetes healthcare setting and the prevalence of various staphylococcal species in multiple distinct anatomical sites of people with diabetes utilising chromogenic media to distinguish distinct species, with and without DFUs.

This project also aims to investigate the oro-nasal cavity as an endogenous reservoir for DFUIs. A microbiological culture-based prevalence approach and whole genome sequencing technology were used to achieve this aim.

Another aspect of this study aimed to investigate the effectiveness of 200 ppm anolyte on the disinfection of debrided DFU tissue to evaluate its effectiveness towards future possible use for DFUI prevention and treatment.

Chapter 2

General Materials and Methods

2.1. Routine isolate culture and storage

Staphylococcal isolates were routinely cultured by aseptically streaking colonies on Sa*Select*™ chromogenic agar (Bio-Rad, Hercules, CA, USA), trypticase soy agar (TSA; Oxoid Ltd., Hampshire, UK), or on Columbia blood agar (CBA; Fannin Ltd., Dublin, Ireland) at 37°C in a Gallenkamp Prime 225 L incubator (Gallenkamp, Cambridge, UK) for 18-24 h.

Purified staphylococcal isolates were maintained in Microbank cryogenic storage beads (MicroBank, Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada) at -80°C for long-term storage. Isolates were reactivated by aseptically removing a single bead from the storage vial and using this bead to inoculate an appropriate agar plate followed by incubation in a static incubator (Gallenkamp) for 48 h at 37°C.

2.2. Patient recruitment

Ethical approval for this project was granted by the Tallaght University Hospital (TUH) and St. James's Hospital (SJH) Joint Research Ethics Committee (REC) on the 9th January, 2020 (Ethics Approval Reference Number 2019-12; Fig. 2.1). The patients recruited to participate in the study were attending the outpatient Diabetes clinics at SJH, provided informed consent and were sampled voluntarily during routine outpatient appointments.

2.3. Chemicals, enzymes and oligonucleotides

Chemicals and molecular grade water, unless specified otherwise, were of analytical grade or molecular biology grade, and were purchased from Merck [Sigma-Aldrich Ltd.], Co. Wicklow, Ireland). Ultra-purified water -generated using a Milli Q Biocel system (Millipore Ireland, Co. Cork, Ireland) was used for the preparation of chemical solutions and buffers. Phosphate buffered saline (PBS) tablets (Oxoid Ltd.) were dissolved in purified Millipore water and sterilised by autoclaving in a Tomy SX-500E autoclave (Tomy Kogyo Co., Ltd., Tokyo, Japan) according to manufacturer's instructions.

Reagents for molecular biology procedures such as polymerase chain reactions (PCRs), including enzymes, dNTPs, DNA loading dye and 5 × green GoTaq® flexi buffer were purchased from the Promega Corporation (Madison, WI, USA). GoTaq® DNA polymerase (Promega) was used in all PCRs.

Tallaght University Hospital | Ospidéal Ollscoile Thamhlachta
An Academic Partner of Trinity College Dublin

SJH/TUH Research Ethics Committee Secretariat
email: researchethics@tuh.ie

Dr Brenda McManus,
Dublin Dental Hosptial,
Lincoln Place,
Dublin 2

09th January 2020

REF: Comparison of Staphylococcal Species from the Oro-Nasal Cavity, Periodontal Pockets and Foot Ulcers of T2DM Patients: Potential Reservoir for Diabetic Foot Ulcer Infection

REC: 2019-12 Chairman's Action (2)
(Please quote reference on all correspondence)

Date of Valid Submission to REC: 05.06.2019
Date of Ethical Review: 03.12.2019
Research and Innovation Application Number: **PLEASE SUBMIT**

Dear Dr McManus,

The REC is in receipt of your recent request to TUH/SJH Research Ethics Committee in which you queried ethical approval for the above named study.

The Chairman, Prof. Richard Dean, on behalf of the Research Ethics Committee, has reviewed your correspondence given **full approval** for this study to proceed. However, please submit the Research and Innovation Number to the JREC, please add the anonymisation to the PIL, Add consent for samples to the PIL and add swabbing of ulcers to the PILs.

Applicants must submit an annual report for ongoing projects and an end of project report upon completion of the study. It is the responsibility of the researcher/research team to ensure all aspects of the study are executed in compliance with the General Data Protection regulation (GDPR), Health Research Regulations and the Data Protection Act 2018. ***Additionally, please note for documents submitted for GDPR purposes that the REC and the Chair are not confirming that you're documents are GDPR compliant, they are approving the document from an ethical perspective.***

Yours sincerely,

Sadhbh O'Neill

REC Officer – Dr Sadhbh O'Neill
SJH/TUH Research Ethics Committee

The SJH/TUH Joint Research and Ethics Committee operates in compliance with and is constituted in accordance with the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004 & ICH GCP guidelines.

Figure 2.1. Chairman's letter from the TUH/SJH Joint Research Ethics Committee **confirming ethical approval for the research project.**

Reagents, buffers and adapters required for the preparation of sequencing libraries were purchased from Illumina (Illumina, Eindhoven, Belgium). Oligonucleotides were purchased from Merck. These oligonucleotides were purchased at 100 nM and were subsequently diluted to a working concentration of 10 nM using molecular biology reagent water purchased from Merck. Purification of PCR products was carried out using the GenElute™ PCR CleanUp Kit (Merck) according to the manufacturer's instructions.

2.4. Nucleic acid extraction

2.4.1. Nucleic acid extraction for polymerase chain reactions (PCRs)

Single colonies were lawned out onto fresh CBA plates and incubated for 24 h at 37°C. A sterile 1 µl inoculation loop (Greiner Bio-One, Kremsmünster, Austria) was used to collect a loopful of each fresh bacterial culture which was was resuspended in a sterile 1.5 ml Eppendorf Safe-lock™ microfuge tube (Eppendorf, Hamburg, Germany) containing 700 µl sterile PBS and approximately 300 µl of sterile acid-washed glass beads (Merck). Cells were disrupted using a bead beater (Mini-BeadBeater 24 Cell Disruptor, BioSpec Products Inc. Bartlesville, OK, USA) at three pulses of 40 s at 6.0 m/s. The tubes were then centrifuged at 18,000 × *g* in a sterile 1.5 ml Eppendorf tube using a model 5430 benchtop centrifuge (Centrifuge 5430, Eppendorf AF, Hamburg, Germany) and 400 µl of the supernatant was transferred into a clean 1.5 ml Eppendorf tube, and 200 µl of ice-cold ethanol was added to precipitate DNA from the supernatant solution. Tubes were then inverted three times to mix. The contents of the tube (including any precipitate) were then transferred into individual Dneasy Mini spin columns supplied in the Qiagen DNeasy® Blood and Tissue kit (Qiagen, Manchester, UK) and centrifuged at 13,000 × *g* for one min. Genomic DNA was isolated from the cell lysate suspension remaining in the Eppendorf tubes using DNeasy® mini spin columns and collection tubes which were supplied in the Qiagen DNeasy® Blood and Tissue kit. Subsequent wash and centrifugation steps were carried out according to the manufacturer's instructions. DNA was eluted into 50 µl of molecular biology reagent water (Merck) and was stored at -20°C prior to subsequent analysis.

2.4.2. Nucleic acid extraction for whole-genome sequencing

Single colonies were lawned out onto fresh CBA plates and incubated for 24 h at 37°C. A sterile inoculation loop (Greiner Bio-One) was used to collect a loopful of each fresh bacterial culture which was resuspended in a sterile 1.5 ml Eppendorf tube containing 200 µl Inter-Array lysis buffer and lysis enhancer (Iter-Array fzmb GmbH, Bad Langensalza, Germany). The cell suspension was vortexed at maximum speed using the Heidolph REAX

top vortex (Heidolph, Schwabach, Germany) for one min and lysis of the bacterial cells was performed by incubation for 3 h at 37°C with shaking in a Cliften NE5-28 shaking water bath (Nickel Electro Ltd., North Somerset, United Kingdom) at 250 rpm. Following lysis of the bacteria, 25 µl of proteinase K (20 µg/ml) and 200 µl of buffer AL (both supplied in the Qiagen DNeasy® Blood and Tissue kit) were added to the Eppendorf tube containing the cell lysate, which were subsequently incubated at 70°C for 30 min to degrade cellular proteins in the cell lysate. Genomic DNA was isolated from the remaining cell lysate suspension using DNeasy® mini spin columns and collection tubes which were supplied in the Qiagen DNeasy® Blood and Tissue kit. Subsequent wash and centrifugation steps were carried out according to the manufacturer's instructions (Qiagen). DNA was eluted into 50 µl of molecular biology reagent water (Merck) and was stored at -20°C prior to subsequent analysis.

2.5. Determination of concentrations and quality of extracted DNA and DNA sequencing reactions

If necessary, the concentration of extracted staphylococcal DNA was increased by evaporation in 1.5 ml Eppendorf tubes in a QBD2 dry heating block (Grant, Cambridgeshire, UK) at 70°C for 30 min with the lids of the tubes open.

DNA concentrations were determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). The concentration and purity of extracted DNA samples was individually assessed based on the OD A260/A280 and OD A260/A230 ratios, respectively. An A260/A280 ratio of approximately 1.8-2.0 and OD A260/A230 ratio 2.0-2.2 was deemed acceptable for subsequent downstream molecular analyses. For general use in PCR, each DNA sample was normalised to 60 ng/µl using ultrapure water (Merck).

2.6. Agarose gel electrophoresis

Electrophoresis buffer was prepared at a 5 × concentration using 0.45 M Trizma base, 0.45 M boric acid, 0.01 M EDTA, (TBE buffer) pH 8. This was diluted to 0.5 × in Milli-Q® purified water (Millipore) for agarose gel electrophoresis.

Agarose gels (2% w/v) were prepared by dissolution of agarose powder (Genetic Analysis Grade Agarose, Broad Separation Range, Fisher Bioreagents, Fisher Scientific, Hampton, NH, USA) in 0.5 × TBE buffer by heating in a Sharp R21-ATP 1000 w microwave oven (Fridgemaster, Dublin, Ireland). Gels were cast in a Galileo Bioscience (Cambridge, MA, USA) electrophoresis system gel tray using gel-spacing well combs (Thermo Scientific, Waltham, MA, USA). GelRed™, used to fluorescently stain DNA samples in agarose gels, was purchased from Biotium (Fremont, CA, USA) and incorporated into agarose gels

immediately prior to casting at a final concentration of 1% (v/v). Agarose gel electrophoresis was carried out for approximately 2-3 h using a Consort model EV222 (B-2300 Turnhout, Belgium) power pack set at 102 V and 80 mA and a Galileo Bioscience (Cambridge, MA, USA) gel electrophoresis system and well combs (Thermo Scientific, Waltham, MA, USA). Visualisation of stained, separated PCR products in agarose gels was perfomed by ultraviolet light illumination at a wavelength of 312 nm using an Alpha Innotech model AVT26U transilluminator (Protein Simple, San Jose, CA, USA) and the AlphaImager mini software (Protein Simple). Gel images were printed using a Mitsubishi (Sant del Vallés, Barcelona, Spain) model P93DW printer.

2.7. Sanger-based DNA sequence determination

Sanger-based sequencing reactions (125) were performed commercially with an ABI 3730xl Sanger DNA analyser and an ABI BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA) by Source Bioscience (Source Bioscience, Cambridge, UK) and Eurofins (Eurofins, Konstanz, Germany).

2.8. Recovery and identification of staphylococcal species

2.8.1. Putative species identification on chromogenic agar

Sa*Select*™ agar plates (BioRad) were utilised for the detection of mixed staphylococcal species from clinical samples and to presumptively identify recovered staphylococcal isolates according to species-specific colony colour following incubation at 37 ºC for 48 h (Fig. 1.2).

2.8.2. Latex agglutination testing for Staphylococcus aureus

Presumptive *S. aureus* isolates recovered on Sa*Select*™ agar were tested with the Pastorex-plus latex agglutination test kit (Bio-Rad, Marnes la Cocquette, France), which detects the fibrinogen-affinity factor (known as clumping factor), protein A, and/or capsular polysaccharides of *S. aureus.* The test is performed by mixing a small loopful of a presumuptive 18 h old *S. aureus* colony into a droplet of the latex test solution. A positive result is indicated by rapid visible agglutination of the cells in the latex test solution.

2.8.3. Definitive species identification by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)

Single bacterial colonies of interest were purified on CBA or TSA and incubated at 37°C for 18 h. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) using the VITEK MS system (bioMérieux, Marcy L'Etoile, France) was

used to definitvely identify isolates, and was performed by Ludmila Fadejeva, Tanya Fleming or Paul Grier at the National MRSA Reference Laboratory, St. James's Hospital, Dublin.

2.8.4. Definitive species identification by sequence analysis of the 16S rRNA gene

Definitive identification of CoNS isolates of interest was also performed based on PCR amplification of genomic CoNS DNA and Sanger-based nucleotide sequence determination of the 16S rRNA gene (126, 127) using the same pair of oligonucleotide primers for both amplication and sequence determination (Table 2.1). Primers for definitive identification of *S. aureus* and *S. epidermidis* (Sa-Se ID primers) were used in the definitive identification of *S. epidermidis* without the need for Sanger-based sequencing of the 16s rRNA gene (Table 2.1). Sanger-based sequencing was performed commercially as described in Section 2.7. Resultant DNA sequences were submitted to the NCBI BLAST search engine (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to comparatively identify isolates based on consensus 16S rRNA nucleotide sequences.

2.9. Whole genome sequencing

Short-read WGS determination and analysis was performed as outlined in Fig. 2.2. Genomic DNA was extracted using the method described previously in Section 2.4.2. Sequencing libraries were prepared utilising the Illumina ILMN DNA LP (M) preparation kit, and were sequenced using the MiSeq v3 cycle reagent kit and a MiSeq desktop sequencer (Illumina). Samples were run in batches varying between 50-60 samples per run. Libraries were scaled to have a minimum of 70 × coverage per individual isolate.

Table 2.1. Oligonucleotide primers used in the identification and molecular typing of staphylococcal species.

Primer	Amplicon size (bp)	Primer sequence (5'→ 3')	Primer target (GenBank accession No.)	Annealing Temperature (ºC)	Reference
27F	1400	AGAGTTTGATCATGGCTCA	16S rRNA	55	Heuer *et al.*, 1997 (128)
1492R		TACGGTTACCTTGTTACGACTT	16S rRNA		
S. aureus ID F	379	GAATTAATGGAAGCTGTAGATACT	*tuf gene* (YP_499102.1)	55	This study
S. aureus ID R		CTTCGTCTTTTGATAATACG	*tuf gene* (YP_499102.1)		This study
S. epidermidis ID F	201	CGGTATGCACGAAACTTCTA	*tuf gene* (YP_499102.1)	55	This study
S. epidermidis ID R		GATAATACGTATACTTCAGCTTT	*tuf gene* (YP_499102.1)		This study

Abbreviations: bp; base pair, ID; identification, F; forward primer, R; reverse primer, rRNA; ribosomal ribonucleic acid.

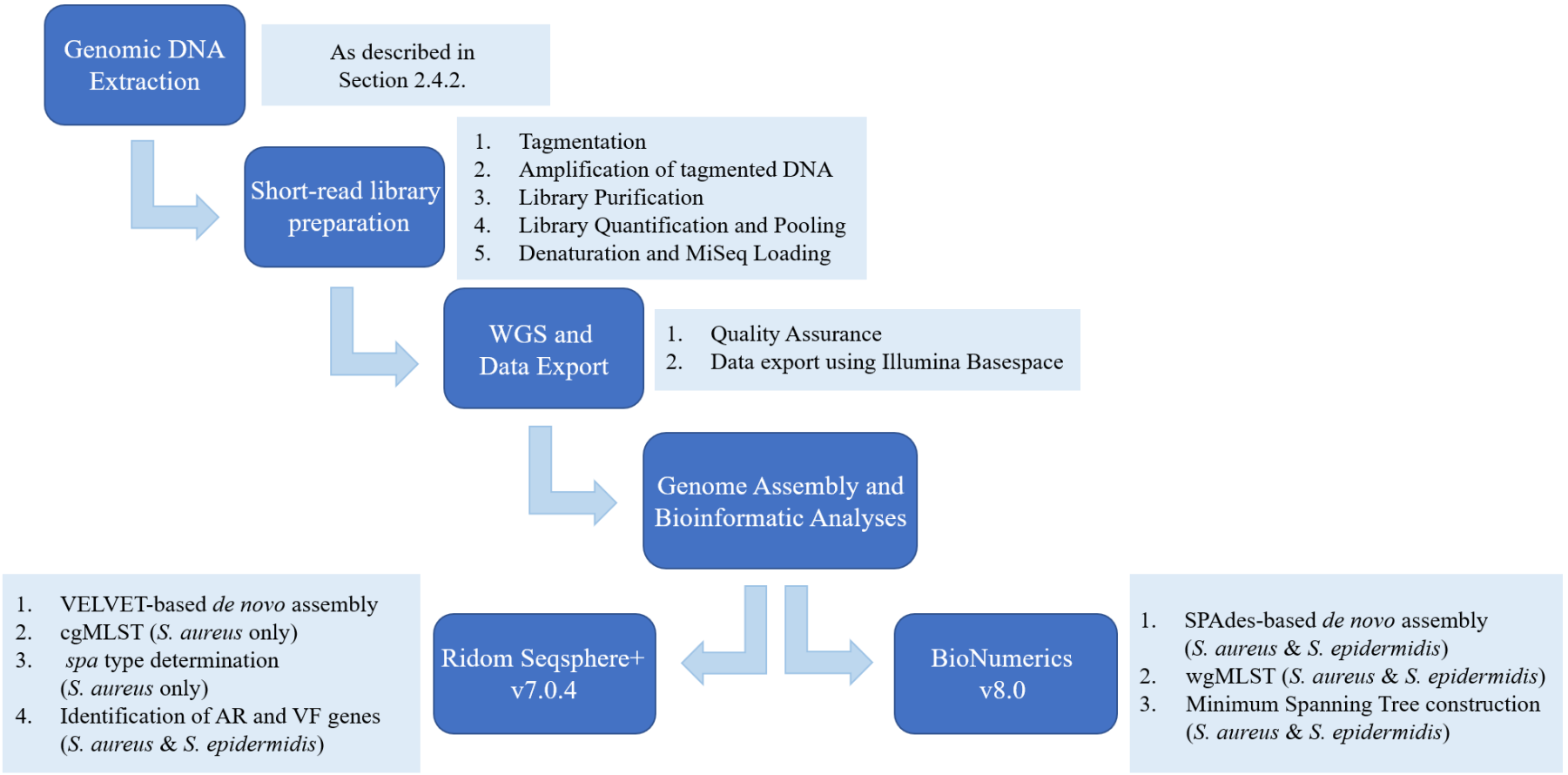


Figure 2.2. Processing workflow for the short-read whole-genome sequencing and data analysis pathway used in the present study. Abbreviations: AR; Antimicrobial resistance, cgMLST; core-genome multilocus sequence typing, wgMLST; whole-genome multilocus sequence typing, SNV; Single Nucleotide Variation, VF; Virulence factor.

For each 15 µl volume containing 250-300 ng of genomic DNA, a master mix of 5.5 µl of bead-linked transposomes (BLTs) and 5.5 µl tagmentation buffer (TB1) (both supplied by Illumina) per sample was prepared according to the manufacturer's instructions. A 10 µl aliquot of this tagmentation master mix was then added to 15 µl of the genomic DNA in separate 0.2 ml PCR tubes (Fisher Scientific™, Dublin, Ireland) for each isolate, followed by an incubation at 55 ºC for five min. Following this step, 5 µl of tagmentation stop buffer (TSB) (Illumina) was immediately added to each tube, followed by a subsequent incubation step of 37ºC for five min. Following incubation, the contents of each tube were transferred to separate wells of a 96-well PCR plate (Fisher Scientific™). The PCR plate was placed on top of a magnetic stand (Fisher Scientific™) for three min to allow the magnetic separation of the BLTs from the supernatant. BLTs were then washed using tagmentation wash buffer (TWB) three times using the magnetic stand to separate the beads from the supernatant between each wash.

2.9.2. Amplification of tagmented DNA

A mastermix of 11 µl of enhanced PCR mix (EPM) (Illumina) and 11 µl nuclease free water (Merck) per sample was prepared. Following the removal of the third wash from the libraries, each DNA sample was mixed with 20 µl of the EPM/water mix, 2.5 µl of an i5 adapter and 2.5 µl of an i7 adapter (Nextera DNA CD Indexes, Illumina). Unique index adapter combinations were used for each sample per sequencing run. Libraries were subjected to amplification using the following incubation steps on the PCR thermocycler; 68ºC for three min, 98ºC for three min, six cycles of 98ºC for 45 s, 62ºC for 30 s and 68ºC for two min, followed by a final incubation step at 68ºC for one min and a 10ºC hold.

2.9.3. Library purification

Library purification was performed using sample purification beads (SPBs) according to the manufacturer's instructions (Illumina). Following the amplification step, the PCR plate was placed on the magnetic stand (Magnetic Stand – 96, Invitrogen, Waltham, MA, USA) for three min. Once the beads had formed magnetised pellets, 22.5 µl of the PCR supernatant was transferred to separate wells of a 96-well Midi plate (Abgene/Fisher Scientific™, Hampton, NH, USA). A mastermix of 22.5 µl SPBs and 20 µl of nuclease free water per sample was prepared and 42.5 µl was added to each sample. The samples were thoroughlymixed by pipetting, incubated at room temperature (RT) for five min and placed back on the magnetic stand for five min. Following the clearing of the SPBs to a pellet within each sample, 62.5 µl of each supernatant was then transferred into new wells of the same

plate, 7.5 µl of undiluted SPBs were then added and the samples were incubated again at RT for five min. Following incubation, the plate was again placed on the magnetic stand for five min. Once clear, the supernatant was removed from each well and discarded, and the beads were washed twice using freshly prepared 80% (vol/vol) ethanol whilst the Midi plate was still on the magnetic stand. The plate was removed from the magnetic stand and the beads in each well were then resuspended in 17 µl resuspension buffer (RSB) (Illumina). Samples were incubated at RT for two min and the Midi plate was again placed on the magnetic stand for two min. Once clear, 15 µl of each supernatant (containing the purified library) was removed and transferred into separate, new 0.2 ml PCR tubes. Samples were stored at -20ºC for up to 30 days.

2.9.4. Library quantification, normalisation and pooling

The nucleic acid concentration of each library weas measured using the Qubit Fluorometer 3.0 (ThermoFisher, Dublin, Ireland) according to the manufacturer's instructions. Samples with concentrations below 4 ng/µl were discarded and the library preparation was repeated for such samples. Following quantification, all library concentrations were adjusted to 10 nM. A 5 µl volume of each prepared library was pooled into one 1.5 ml Eppendorf safe lock tube. Up to 60 libraries were pooled for WGS runs using MiSeq v3 Reagent Kits and cartridges (Illumina). The concentration of each pooled library was confirmed using the Qubit Flurometer and diluted to 4 nM using nuclease free water (Merck). Pooled libraries were stored at -20 ºC for up to 7 days.

2.9.5. Final denaturation, MiSeq loading and run quality assurance

Pooled libraries were subjected to a denaturation step which consisted of adding 5 µl of 0.2 N NaOH to 5 µl of the 4 nM pooled library in a fresh Eppendorf tube. This tube was incubated at RT for five min. Following incubation, the denatured pool was diluted to a 20 pM concentration using prechilled HT1 hybridisation buffer (Illumina).

Immediately prior to MiSeq loading, 360 µl of the 20 pM denatured libraries were combined with 240 µl prechilled HT1 resulting in a 12 pM denatured library. A 6 µl volume of the library was removed, discarded and replaced with a 6 µl aliquot (1% spike) of PhiX control library (Illumina). To ensure complete separation of DNA strands, the denatured library was incubated at 96ºC for two min, followed by incubation on ice for five min. A 600-cycle MiSeq Reagent v3 kit was used to perform paired-end sequencing on the pooled, normalised and denatured DNA libraries on the Illumina MiSeq instrument according to the manufacturer's instructions. Following the completion of each MiSeq run, the run quality

was determined in accordance to cluster density and quality score (Q30) on the instrument or on the Illumina BaseSpace cloud (https://basespace.illumina.com).

2.9. Bioinformatic Analyses

2.9.1. Genome assemblies

Following a sequencing run, the FASTQ files generated were exported from the MiSeq onto the web via Illumina BaseSpace cloud (https://basespace.illumina.com/). These files were then imported for bioinformatic analysis using the BioNumerics Genome Analysis Tool (GAT) plug-in version 8.0 (Applied Maths, Sint-Martens-Latem, Belgium). The BioNumerics v8.0 software and incorporated SPAdes assembly software v3.7.1 (Applied Maths) were used to perform *de novo* assemblies. The quality statistics in-built tool within the BioNumerics v8.0 (Applied Maths) software was used to assess the quality assurance of sequence reads, *de novo* assemblies, assembly-free and assembly-based calls. Sequence reads predicated on their average read quality according to the manufacturer's instructions (average score >30). The *de novo* assemblies were assessed based on the length of the median contig (N50 >100,000).

The short-read FASTQ datasets were also imported into Ridom Seqsphere+ v7.0.4 (Ridom GmBH, Münster, Germany) and assembled using the VELVET genome assembly tool (v1.2.10). Quality filtering was undertaken with SeqSphere+ (Ridom) and trimmed reads were subjected to *de novo* assemblies using default parameters.

2.9.2. Core-genome multilocus sequence typing (cgMLST)

Population-based studies were performed with SeqSphere+ v7.0.4 (Ridom) using the previously described *S. aureus*- and *S. epidermidis*-specific core-genome multilocus sequence typing (cgMLST) schemes (129). *The S. aureus* cgMLST scheme consists of 1,861 conserved housekeeping genes within the *S. aureus* core genome and also includes the original seven housekeeping loci previously used for conventional multilocus sequence typing (130) (MLST). The *S. epidermidis* cgMLST scheme consists of 2,058 core loci conserved housekeeping genes within the *S. epidermidis* core genome and also includes the seven housekeeping loci previously used for conventional MLST (129, 131).

2.9.3. Whole-genome multilocus sequence typing (wgMLST)

Whole-genome multilocus sequence (wgMLST) typing was performed for both *S. aureus* and *S. epidermidis* using BioNumerics v8.0 (Applied Maths) software. The *S. aureus* wgMLST scheme within BioNumerics v8.0 software was designed to analyse 3,904 gene

loci, thus providing higher typing resolution. This included the previously defined 1,861 core genome housekeeping genes (129) as well as *S. aureus* accessory loci. The *S. epidermidis* wgMLST scheme within BioNumerics v8.0 software was designed to analyse 4,877 gene loci, which also provides higher typing resolution which includes the 2,058 core genome housekeeping genes, as well as the *S. epidermidis* accessory loci.

Short-read sequencing datasets were subjected to two different automated pipelines for each species. Firstly, the incorporated SPAdes assembly software v3.7.1 within BioNumerics v8.0 (Applied Maths) was used to perform assembly-free allele calling on the sequence reads directly, to determine which loci were present/absent and confirm MLST profiles. The software used a *k*-mer approach (pairwise comparison of a set of genomes based on all the possible subsequences/substrings of length '*k*' in the sequence data), with a default *k*-mer length of 35 bases applied to all sequence reads. Only loci with a minimum total coverage of 3× (minimum forward and reverse coverage of 1×) were retained for wgMLST profiling, as per BioNumerics v8.0 (Applied Maths) recommendations.

Secondly, a BLAST search was carried out on *de novo* assembled genomes of the study isolates against the alleles of the seven classical MLST housekeeping genes and 31 publicly available reference genome sequences. This approach is known as assembly-based calling. The default minimum similarity threshold of 80% to the reference sequence was applied, and gapped alignments were allowed. When more than one allelic variant was detected within the loci of one specific sample, it was excluded from the consensus wgMLST profile of that sample.

2.9.4. Minimum Spanning Trees

Minimum spanning trees (MSTs) were generated based on distance matrices and were constructed to visualise the relatedness of isolates and allelic differences between related isolates based on with cgMLST or wgMLST datasets using BioNumerics v8.0 (Applied Maths) and including permutation resampling (1000 replicates). Branch differences indicated the allelic differences between species, where the MST algorithm calculated the shortest distance between isolates, and isolates were visually represented via circular nodes. The previously recommended thresholds of <24 allelic differences determination of the genetic relatedness of both *S. aureus* and *S. epidermidis* were applied to all MSTs. Within these thresholds, 0-24 allelic differences or 0-15 SNVs between isolates were considered as highly related or genetically identical (132).

2.9.5. Identification of spa *types and antibiotic resistance and virulence factor genes*

Traditional *S. aureus* MLST STs and *spa* types were all determined from the assembled WGS datasets *in silico* using specific corresponding task template tools embedded within the Ridom SeqSphere+ program v 7.0.4. The presence of antibiotic resistance and virulence factor encoding genes in both *S. aureus* and *S. epidermidis* datasets was detected using corresponding Ridom SeqSphere+ v7.0.4 task template tools.

Chapter 3

Immersion of debrided diabetic foot ulcer tissue in electrochemically generated pH neutral hypochlorous acid significantly reduces the microbial bioburden: whole-genome sequencing of *Staphylococcus aureus*, the most prevalent species recovered

3.1. Introduction

3.1.1. Diabetic foot ulcer infections

Diabetic foot ulcers (DFUs) and associated disease are a devastating consequence of diabetes which can have lifelong implications for patients and their care support networks. Approximately 50-60% of DFUs (133) develop DFU infection (DFUI), increasing the risk of hospitalisation and LEA by 56- and 155-fold, respectively (134). The development of DFUIs requires more frequent healthcare visits, increased healthcare costs and increases the risk of LEA and morbidity (135). Approximately 20% of DFUIs result in LEA (136). All DFUIs primarily develop as superficial infections, predominantly caused by aerobic Gram-positive bacteria such as staphylococci and β-haemolytic streptococci. *Staphylococcus aureus* is the predominant bacterial species recovered from DFUIs (137, 138). As the infection progresses, bacterial loads increase and populations diversify which typically leads to the formation of deeper, chronic polymicrobial infections (139). These are more difficult to treat due to the development of microbial biofilms in active ulcer sites, which are less responsive and often resistant to antibiotics treatments (28). Bacterial biofilm presents the number one cause for delayed healing in chronic wounds. Biofilm prolongs the presence of innate immune cells, including neutrophils and macrophages, impairing wound healing and potentially reducing the effectiveness of the innate immune response (140). Hospitalisation and surgical procedures, as well as failure to complete prescribed courses of antibiotics, can result in colonisation and infection by antibiotic resistant microorganisms such as methicillin-resistant *S. aureus* (MRSA) or vancomycin-resistant enterococci (VRE) (141). Between 16.7% and 44.4% of DFUI-associated *S. aureus* populations are MRSA (142-144).

Severe DFUIs that necessitate LEA are usually polymicrobial and predominantly include organisms such as β-haemolytic streptococci, enterobacteriaceae, *Pseudomonas aeruginosa*, and enterococci. Anaerobic organisms, such as bacteroides, peptococcus, and peptostreptococcus, are often present, however rarely are the sole cause of infection, and exist in deep soft tissue infections and osteomyelitis (145). Targeted amplicon sequencing has confirmed the polymicrobial nature of DFUIs, of which specific microbial population compositions can influence treatment outcomes, including heightened difficulty in managing the wound and infection, and possible LEA (30, 146, 147). Barshes *et al.* identified 156 unique species from 727 specimens obtained from initial foot infection from 694 patients with DFUs, and found that the presence of *S. aureus* is negatively associated with that of other staphylococci and is more likely to be isolated by itself (146). Malone *et*

al. reported that DFUs of less than six weeks duration typically harboured predominantly one organism, either *S. aureus* or *Streptococcus agalactiae*. In contrast, DFUs of longer durations harboured diverse microbial populations and severe DFUIs were found to harbour highly complex microbiomes, characterised by multiple distinct species present in low abundance (147). Smith *et al.* described the presence of 41 unique genera comprising 81 distinct strains amongst the DFUs of 20 patients. Swab samples of these DFUs were subjected to culture-based microbiological analysis under both aerobic and anaerobic conditions. Amplicon sequencing of the 16S genes was used to definitively identify bacterial isolates in the culture-positive samples. *Staphylococcus aureus* was identified in 72%, while the most commonly identified strains from all cultures were *Peptoniphilus* spp., *Anaerococcus* spp. *and Corynebacterium* spp. The majority of species residing in new and recurrent ulcers in this cohort of patients were Gram-positive cocci organisms, such as *Staphylococcus, Streptococcus, Anaerococcus, Peptoniphilus* and *Finegoldia*, the latter three of which are anaerobic organisms (30). Although wounds are frequently exposed to air, anaerobes can survive via co-aggregation with facultative anaerobic organisms or aerobes within a polymicrobial biofilm structure within the wound. This biofilm can protect anaerobic organisms from harmful effects of oxygen, allowing them to grow and thrive (148). The large quantity of data suggesting polymicrobial wounds containing both aerobic and anaerobic organisms are of the highest risk of developing severe infection warrants thorough investigation of the role of anaerobic organisms in chronic wounds.

The underlying ulcer aetiology may also influence the microbial populations associated with DFUIs. For instance, patients with ischaemic ulcers are more likely to be infected with anaerobic bacterial species such as those belonging to the *Clostridia, Peptostreptococcus, Bacteroides, Porphyromonas* and *Veillonella* genera (149, 150). Haldar *et al.* described the microbial ecologies of polymicrobial ulcer infections, characterising anaerobic and aerobic populations. Swab samples were collected from patients undergoing routine debridement therapy. Where possible, tissue and bone samples were also collected for processing. All of the 43 samples investigated were culture-positive, harbouring predominantly anaerobic Gram-negative organisms including *Bacteroides, Porphyromonas, Veillonella* species and *Clostridium perfringens*, as well as anaerobic Gram-positive organisms such as *Peptostreptococcus*, followed by aerobic Gram-positive organisms, of which *S. aureus* was the most predominant organism detected (150). Neuropathic non-ischemic ulcers also harbour diverse microbiomes, as outlined previously by Gardener *et al.* [20]. This study investigated swab samples of 52 neuropathic DFUs, and found that ulcer

depth and duration are associated with microbial biodiversity, where deeper and longer lasting ulcers contained a more diverse microbiome, containing high levels of anaerobic organisms, including *Porphyromonas, Anaerococcus, Finegoldia, Peptoniphilus, Prevotella*, bacteria of the order Bacillales, which include the genera *Bacillus, Listeria and Staphylococcus,* and bacteria of the phylum Proteobacteria. Superficial ulcers harboured higher levels of staphylococci and streptococci, particularly *S. aureus*, which was negatively associated with deeper wounds (151).

3.1.2. Diagnosis and management of DFUIs

The precise role of microorganisms in wound colonisation versus DFUI development has long been debated. Many researchers and medical practitioners recommend a quantitatively based DFUI diagnosis in addition to a clinically based diagnosis of infection, the latter of which is made according to local or systemic signs of inflammation such as purulent secretions (pus), swelling, redness, warmth and pain or tenderness. Physical examination of the wound can be limited by individual clinician subjectivity and the lack of quantitative data regarding microbial bioburden, or the presence of biofilm (152). In its latest 2023 update, The International Working Group on the Diabetic Foot (IWGDF) recommended that infections be diagnosed based on at least two clinical symptoms of inflammation, or the detection of purulent secretions from the wound. Infections should be graded as follows: mild (superficial ulcer with minimal cellulitis); moderate (ulcer deeper than skin or more extensive cellulitis, with or without abcess); or severe (accompanied by systemic signs of sepsis) with or without osteomyelitis (153). These guidelines do not include quantitative microbial bioburden investigation of the wound. Previous research indicated that a DFU bacterial bioburden of $\geq 10^5$ colony forming units per gram (CFU/g) of tissue impedes wound-healing (154). The lack of quantitiative microbiological investigation of DFUs likely has important implications for the underdiagnosis of infection in immunocompromised populations such as those with diabetes.

Early diagnosis and successful DFUI treatment is crucial to prevent further spread of infection into soft tissues and to bone, which may ultimately necessitate LEA (155). Currently, the gold standard for DFU management includes debridement of necrotic tissues, infection control, revascularisation procedures and off-loading weight off the ulcer (156). Antibiotics used to treat infection should include broad-spectrum antibiotics which kill mixed aerobic (*S. aureus, Enterococcus* spp., *Streptococcus* spp., *Escherichia coli*) and anaerobic (*Bacteroides fragilis, Clostridium perfringens* and *Peptostreptococcus* spp.)

populations. Moderate infections should be treated with oral antibiotics including cephalexin, amoxicillin, moxifloxacin, or clindamycin. More severe polymicrobial infections should be treated with oral or intravenous antibiotics (157). For these severe infections, oral or intravenous antibiotics are typically prescribed instead of topical antibiotics, including anti-pseudomonal penicillins, broad-range penicillins, fluoroquinolones, cephalosporins or carbapenems (158).

3.1.3. Electrochemically activated (ECA) solutions

3.1.3.1. Generation of ECA solutions

Electrochemically activated solution (ECA) technology was developed as a specialised discipline of electrochemistry by Professor Vitold Bakhir in the former Soviet Union in the 1970s (101). ECA solutions are generated by passing a dilute salt solution through an electrical field in a flow-through electrolytic module (FEM) and separating the reactive solutions generated by an ion-selective membrane changing the salt solution from a stable to a metastable state (Fig. 3.1). The two oppositely charged and separated solutions possess different physical and chemical properties (159). The positively charged solution, termed anolyte, consists predominantly of metastable hypochlorous acid [HOCl] and other unstable mixed oxidants including hypochlorite [OCL^-] held together in a physically excited state and has a redox value of approximately 600-900 mV depending on the ECA generator and pH. Hypochlorous acid is a powerful antimicrobial oxidant that is naturally produced by neutrophils. The negatively charged antioxidant solution, catholyte, is composed primarily of sodium hydroxide [NaOH] and has detergent properties. Catholyte typically has a pH of 11 and a redox value of -600-900 mV.

The generation of ECA solutions is not universally consistent. Different kinds of ECAs have been designed following the development of this technology and are produced in different types of generators with specific properties. For example, their generation may not include membrane separation and are more akin to hypochlorite solutions.

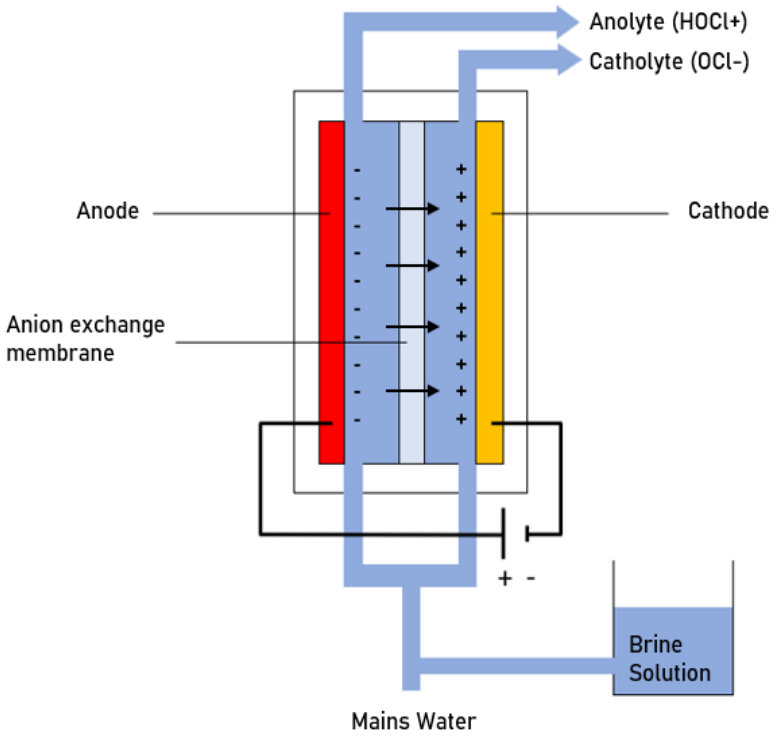


Figure 3.1. A schematic diagram of a flow-through electrolytic module (FEM). A mixture of water and dilute 0.2% sodium chloride are passed through the electrolytic cell. The FEM is composed of a set of electrodes, the anode and cathode, separated by a semi-porous membrane known as the anion exchange membrane. During electrolysis, negative ions migrate to the anode and the predominant product formed is hypochlorous acid, while positive ions migrate to the cathode and the predominant product formed is sodium hydroxide. The arrows in the centre of this diagram indicates the passing of the brine solution through the anion exchange membrane where ions migrate to the anode or the cathode. The two metastable solutions produced, anolyte (positively charged) and catholyte (negatively charged) are exported separately (Figure adapted from Swan *et al.*, 2016 (166)).

Anolyte produced by older model ECA generators tend to produce acidic anolyte solution which is highly corrosive (160). Modern ECA generators such as those produced by the Trustwater Group (Clonmel, Ireland) and Clarentis (Clarentis Technologies, FL USA) can be configured to produce pH neutral anolyte solutions that are environmentally friendly, non-toxic, inexpensive, highly microbicidal, sporicidal and capable of penetrating biofilms (137, 161). Highly to moderately acidic anolyte solutions have higher concentrations of HOCL and a higher redox potential, but as mentioned above tend to be corrosive. These ECA solutions stored over time in bottles have the potential to lose physiochemical and bactericidal properties in comparison to freshly generated ECAs. Thorn *et al.* found that ECAs stored in glass and polystyrene containers, which were monitored for FAC, ORP, pH and bactericidal activity, lost ORP and FAC levels declined over time. These solutions declined most rapidly at 20°C in polystyrene bottles and deteriorated least when stored in glass at 4°C. Bactericidal efficacy was also greatly reduced, and ECAs stored at 20°C failed to produce a 5 log10 reduction on five separate occasions when tested on *P. aeringosa* on tryptone soya agar. The pH was the only constant to not change significantly in stored ECAs (162).

Numerous studies over the past three decades have shown that output water from dental unit water lines (DUWLs) is heavily contaminated with microorganisms which can form thick biofilm (106, 159, 163-165). Electrochemically-activated solutions have been shown to prevent contamination and biofilm formation in dental chair units and DUWLs. One study showed that the average contamination of microorganisms in unprocessed mains water and anolyte-treated water used to supply DUWLs was 88 c/ml and <1 CFU/ml, respectively, (159). It was shown, more recently, in another long-term (one year) study, that microbial contamination of clinical washbasin output water (both hot and cold) and associated taps can consistently be minimised by residual treatment of supply water with anolyte (111). Biofilm present in hospital wastewater pipes and U-bends have also proven to be difficult to eradicate (112). U-bends are wet, nutrient-rich segments of wastewater pipework and are frequently coated in dense biofilm. Automated U-bend decontamination systems have been used to remove these biofilm-forming micro-organisms from hospital U-bends. Two recent studies demonstrated significant reductions in U-bend microorganisms and biofilm following sequential treatment of U-bends with catholyte followed by anolyte ECA solutions relative to non ECA-treated U-bends (166, 167). Electrochemically activated solutions have also been used as a disinfectant in Russia for over 30 years for the disinfection

of drinking water, swimming pools, hospital water lines, for irrigating wounds and for destroying microbial biofilms (160).

3.1.3.2. Application of ECA solutions to wounds

A comprehensive review of the antimicrobial efficacy and mode of action of hypochlorous acid and its use for therapeutic support of infections was published recently (168). This review summarised many previous studies that have demonstrated the efficacy of hypochlorous acid for bacterial bioburden reduction and promotion of wound and burn healing by repetitive irrigation (169, 170).

Anolyte-soaked wound dressings and sprays have shown promise in post-surgical management of DFUIs, promoting healing, reducing bacterial load, odour, and re-infection rates (171, 172). Martínez-De Jesús *et al.* investigated the effects of ECAs on 45 patients who presented with deep, infected wounds. Patients in a control group underwent the conventional treatment of topical antiseptics, while the DFUs of the patients in the test group were immersed in super-oxidised solutions for 15-20 minutes. Compared with conventional disinfectants, the ECA was an effective antiseptic agent with greater odour reduction, greater infection reduction and less tissue toxicity (172). Previous studies investigating the use of hypochlorous acid in a clinical setting were subject to variability including the onset of clinically based infection symptoms, the inclusion of patients already undergoing antibiotic treatment and other therapies, and variability associated with patient-specific immune responses. Furthermore, other clinically-based comparative wound treatment studies have varied in regards to anolyte pH, storage times and conditions which are likely to confound results (173).

3.1.3.3. Safety of ECA solutions

Topical antiseptics including sodium hypochlorite, acetic acid and povidone-iodine are known to be toxic to many of the cells involved in the wound healing process, thus their use on chronic ulcers such as DFUs is now generally discouraged (168, 174). In comparison, hypochlorous acid-based solutions have no cellular toxicity when used in clinically effective doses. Morita *et al.* reported a lack of cytotoxic effects of drinking ECA solutions on the gastrointestinal tracts of mice (175). A separate study revealed that hypochlorous acid was compatible with cells cultured from the umbilical vein up to concentrations of 1,300 (parts per million (ppm) (176). Other research has described high antibacterial activity and lack skin irritation using hypochlorous acid at 250 ppm (177), however more concentrated solutions of 2,500 ppm resulted in weak or mild skin irritations (178). All three phases of

wound healing at the cellular level have been shown to be positively influenced by hypochlorous acid solutions which exhibits anti-inflammatory/infective, cell proliferative and immunomodulatory properties *in vitro* (179).

3.1.4. Study Aims

As kidney disease and ischaemia are common diabetes-related complications which limit the safety and success of systemically distributed antibiotics, the use of alternative inexpensive, topical disinfectants has significant potential benefits. Hypochlorous-acid generated from ECAs has the ability to penetrate and denature biofilm, facilitating HOCl access to wound-resident bacteria (180). A multitude of clinically-based studies demonstrating wound improvement following anolyte treatment suggests the high suitability of these solutions for successful chronic wound and DFU treatment (168-172).

The present study design aimed to eliminate confounding factors typically found in clinical wound treatment studies. It specifically focused on the effectiveness of anolyte in reducing bioburden in debrided DFU tissue *ex vivo*. This approach allowed for a more accurate assessment of the disinfection capacity of HOCl present in anolyte.

This research sought to investigate the consistent effectiveness of pH-neutral anolyte (hypochlorous acid) at reducing microbial loads in debrided DFU tissue, to determine the range of microbial species present in the ulcers and analyse the population of the most prevalent species detected, *S. aureus*, by whole-genome sequencing.

3.2. Materials and Methods

3.2.1. Patient recruitment

Consenting patients with type II diabetes undergoing routine DFU debridement at the St. James's Hospital Dublin (SJH) foot clinic between January 2020 and January 2023 were invited to participate in the study (Appendix 1). Participants were all more than 18 years of age, capable of providing informed consent, had type II diabetes and at least one active DFU requiring routine debridement therapy. Ethical approval was granted by the SJH and Tallaght University Hospital Joint Research Ethics Committee (Approval No. 2019-12) (Fig. 2.1). Multiple debrided tissue samples from the same individuals were collected a minimum of two weeks apart. The dates of tissue collections have been omitted from the study to protect participant anonymity.

3.2.2. Ulcer examination and debrided ulcer tissue collection

A specialist clinical podiatrist performed a brief ulcer assessment using the Texas wound classification scheme (181), recorded clinical ulcer features (Table 3.1) and any prior DFU-related interventions such as surgical debridement, amputation, or antibiotic usage, and collected debrided tissues.

3.2.3. Anolyte

Anolyte was generated daily using a Clarentis Min-Lyte UL-75A (Clarentis Technologies, LLC, Tallinn, Estonia) supplied with mains water and 0.2% (w/v) NaCl solution. The generator was configured to produce anolyte at pH 7.0 with 500 ppm free available chlorine (FAC) consisting of hypochlorous acid (HOCl) (350 ppm) and hypochlorite ion (OCL-) (125 ppm) and an oxidation-reduction potential (ORP) of approximately +880 mV. The FAC concentration of freshly-generated anolyte was determined using a Hach colorimeter (Hach, Western Industrial Estate, Dublin, Ireland) and adjusted to 200 ppm in sterile water generated by a Milli-Q® water purification system (Merck-Millipore) prior to disinfection of tissue samples. Under the EU Biocides Regulation 528/2012 (182) and Commission Implementing Regulation (EU) 2021/364 of 26 February 2021 (183), the anolyte solution used in this study would be classed as active chlorine generated from sodium chloride by electrolysis as an active substance for use in biocidal products of Product Type 1 - human hygiene.

Table 3.1. Clinical features of ulcers from which debrided tissue samples were collected

Sample	Patient No.[a]	Ulcer type [b]	Clinical signs of infection [b]	Saline culture $>10^5$ CFU/g [c]	No. of ulcers	Age of oldest ulcer (weeks)	Amputation history	Ulcer type
Ano1	P1	1A	No	No	4	6	No	Neuropathic
Ano2	P2	1A	No	No	1	1	No	Neuropathic
Ano3	P3	1A	No	Yes	1	0	No	Neuropathic
Ano4	P4	1A	No	Yes	1	14	No	Neuropathic
Ano36	P4	1A	No	Yes	2	2	No	Neuropathic
Ano38	P4	1A	No	Yes	2	4	No	Neuropathic
Ano45	P4	1A	No	No	1	6	No	Neuropathic
Ano5	P5	3D	Yes	Yes	1	72	No	Neuroischaemic
Ano6	P6	1D	Yes	Yes	1	1	No	Neuroischaemic
Ano7	P7	1C	No	Yes	2	8	No	Neuroischaemic
Ano8	P8	1A	No	Yes	3	16	No	Neuropathic
Ano10	P9	1A	No	Yes	1	56	No	Neuropathic
Ano12	P10	1B	Yes	Yes	1	48	Yes	Neuropathic
Ano31	P10	1A	No	Yes	1	14	Yes	Neuropathic
Ano14	P11	1A	No	Yes	1	1	No	Neuropathic
Ano15	P12	3C	No	Yes	1	200	No	Neuroischaemic
Ano16	P13	1C	No	Yes	1	0	No	Neuroischaemic
Ano17	P14	1C	No	Yes	1	12	No	Neuroischaemic
Ano18	P15	1A	No	Yes	1	4	No	Neuropathic
Ano19	P16	1A	No	Yes	2	500	No	Neuropathic
Ano26	P16	1D	Yes	Yes	1	8	No	Neuroischaemic
Ano20	P17	1A	No	Yes	1	5	Yes	Neuropathic
Ano28	P17	1A	No	Yes	1	9	Yes	Neuropathic
Ano37	P17	1A	No	Yes	1	12	Yes	Neuropathic
Ano49	P17	1A	No	No	1	14	Yes	Neuropathic
Ano21	P18	1A	No	Yes	1	20	Yes	Neuropathic
Ano30	P18	1A	No	Yes	1	26	Yes	Neuropathic
Ano48	P18	1A	No	No	1	30	Yes	Neuropathic
Ano13	P19	1A	No	Yes	1	13	Yes	Neuropathic
Ano27	P19	1A	No	Yes	4	10	Yes	Neuropathic
Ano50	P19	1A	No	No	1	14	Yes	Neuropathic
Ano9	P20	1A	No	Yes	1	2	Yes	Neuropathic
Ano25	P20	1A	No	Yes	1	52	Yes	Neuropathic
Ano41	P20	1A	No	No	1	104	Yes	Neuropathic
Ano51	P20	1A	No	No	2	1	Yes	Neuropathic
Ano32	P21	1A	No	Yes	1	40	No	Neuropathic
Ano34	P21	1A	No	Yes	1	43	No	Neuropathic
Ano11	P22	1A	No	Yes	1	17	Yes	Neuropathic

Ano22	P22	1A	No	Yes	2	6	Yes	Neuropathic
Ano43	P22	1A	No	Yes	2	24	Yes	Neuropathic
Ano39	P23	1A	No	Yes	2	6	No	Neuropathic
Ano44	P23	1A	No	Yes	2	9	No	Neuropathic
Ano24	P24	1A	No	Yes	1	20	No	Neuropathic
Ano40	P24	1A	No	Yes	1	46	No	Neuropathic
Ano33	P25	2A	No	Yes	1	16	Yes	Neuropathic
Ano35	P26	1A	No	Yes	2	4	No	Neuropathic
Ano29	P27	2A	No	No	1	20	Yes	Neuropathic
Ano23	P28	1A	No	Yes	1	6	No	Neuropathic
Ano42	P29	1C	No	Yes	1	28	Yes	Neuroischaemic
Ano46	P29	1C	No	Yes	1	4	Yes	Neuroischaemic
Ano47	P30	1A	No	Yes	1	24	Yes	Neuropathic

[a] Multiple debrided tissue samples were collected from patients P4, P10, P16, P17, P18, P19, P20, P21, P22, P23, P24 and P29 at intervals that were a minimum of two weeks apart. Sample collection dates have been omitted from this dataset to preserve participant anonymity.

[b] Wounds were graded and classified according to the Texas Wound Classification scheme and clinical indicators of infection were recorded as previously described (19).

[c] Only 4/42 of the samples that yielded microbial loads $>10^5$ CFU/g, a threshold previously shown to impede wound healing (154), exhibited clinical signs of infection.

3.2.4. Microbiological Processing

Tissue samples were divided into two equal aliquots by wet weight (range 35-75 mg). Paired aliquots were immersed in equal volumes of either freshly generated anolyte or sterile saline for three min (Fig. 3.2). Previous research revealed that the FAC in anolyte rapidly reduces upon contact with organic material (161) and therefore two distinct treatment volumes were investigated; 1 ml (Samples Ano1-Ano20) and 10 ml (Samples Ano21-Ano51) to determine if an excess of anolyte further reduced microbial tissue loads. Tissue samples were exposed to anolyte in 1 ml and 10 ml volumes for three minutes. Following immersion, tissues were recovered by centrifugation at 1520 × *g* (Eppendorf model 5430, Hamburg, Germany) and the supernatant discarded. Residual FAC was neutralised by immersion of tissues in 0.5% (w/v) sodium-thiosulphate for three minutes, following by washing and resuspension of tissues in sterile saline (Fig. 3.2). Resuspended tissues were incubated in the final volume of saline for 10 min at room temperature with shaking at 200 rpm to release any remaining tissue-bound microorganisms into the supernatant prior to microbiological culture (Fig. 3.2).

3.2.4.1. Determination of microbial loads in tissues

An aliquot of supernatant from each tissue sample was diluted ten-fold in saline. Aliquots (100 µl) of each neat and diluted supernatant sample were used to inoculate duplicate Columbia blood agar (CBA) plates that were incubated at 37ºC in a static incubator under aerobic conditions, anaerobic conditions using a GasPak™ EZ anaerobic system (Fisher Scientific, Dublin, Ireland) and on staphylococcal-selective chromogenic Sa*Select*™ (BioRad, Dublin, Ireland) agar (Fig. 3.2). Following incubation, CFUs present on each plate were counted and residual microbial loads in tissues were determined as CFU/g. Microbial load fold-reductions in anolyte-treated tissues relative to saline-treated tissues were calculated for all media and incubation conditions.

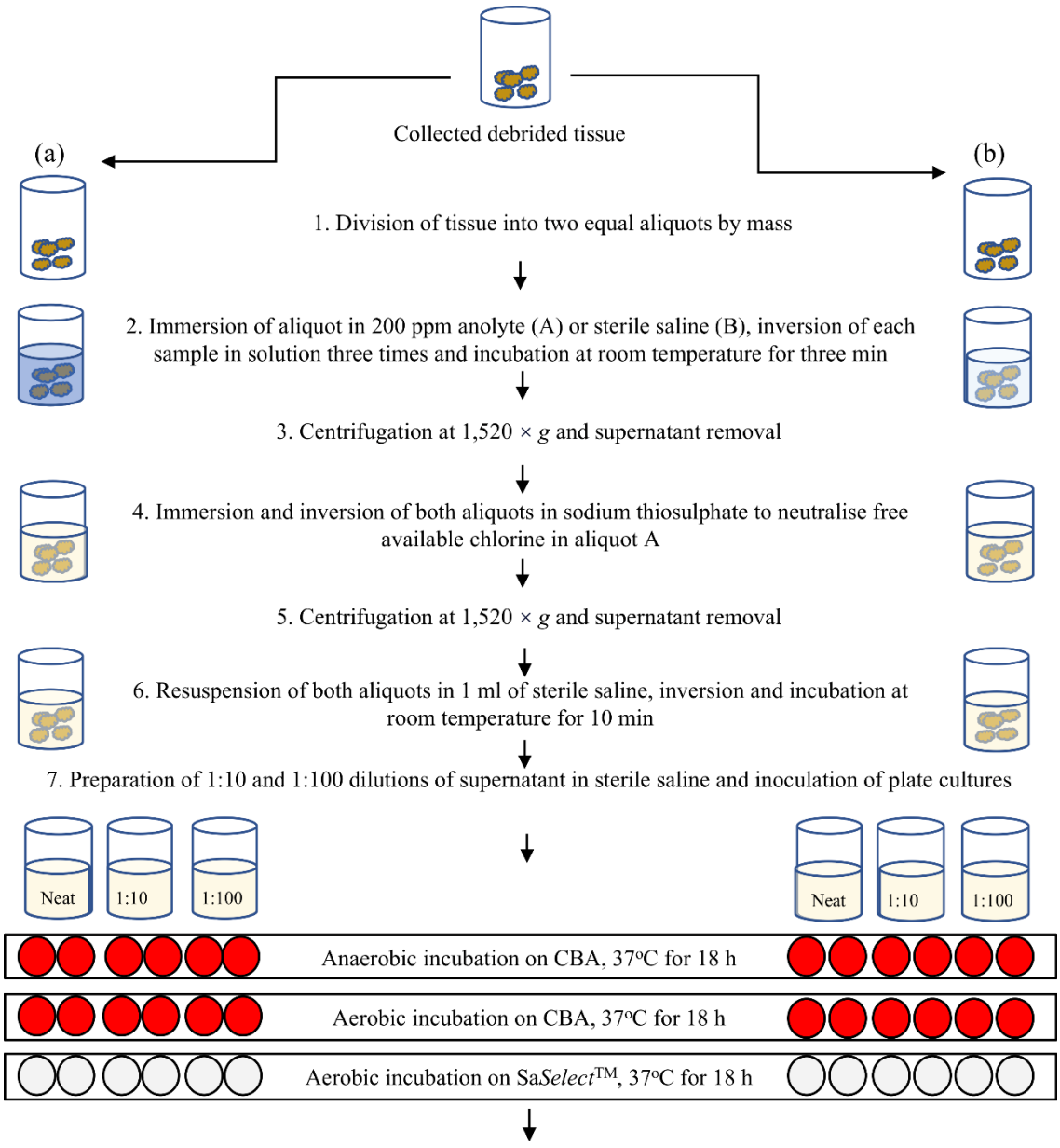


Figure 3.2. Comparative immersion treatment and microbiological analyses of debrided tissue aliquots in (a) 200 ppm anolyte or (b) sterile saline. Debrided tissues were divided into two aliquots based on wet weight which ranged from 35-75 mg. One aliquot was immersed in freshly-generated 200 ppm anolyte, the other in an equal volume of sterile saline. Each tube was inverted three times to maximise contact between the tissue and the treatment liquid and was incubated at room temperature (RT) for three min. Following incubation, each sample was subjected to centrifugation at 1520 × *g* in an Eppendorf model 5430 centrifuge for two min. The supernatant was discarded and the anolyte/saline treatment step was repeated three times. Following the third treatment step, tissues were immersed in sodium thiosulphate (0.5% [w/v]), inverted five times to mix and incubated at RT for three min to

neutralise any remaining free available chlorine (FAC). Samples Ano1-Ano20 were immersed in 1 ml volumes of anolyte/saline and sodium thiosulphate, whereas samples Ano20-Ano51 were immersed in 10 ml treatment volumes. Following immersion in sodium thiosulphate tissues were recovered by centrifugation as above, immersed in 1 ml sterile saline, inverted five times to mix and incubated at RT with shaking for 10 min. The supernatant from each tissue sample was subjected to two 10-fold dilutions from which 100 µl aliquots were used to inoculate duplicate CBA plates that were incubated under aerobic conditions, anaerobic conditions and on the staphylococcal-selective chromogenic agar medium Sa*Select*™. Following incubation, the bacterial density in CFU/g of starting tissue was recorded and the fold reduction in anolyte-treated tissue samples incubated under aerobic, anaerobic and staphylococcal-selective conditions was calculated and recorded.

3.2.5. Isolate storage and identification

Isolates were stored at –80°C on Microbank cryogenic bead vials (Pro-Lab Diagnostics) as described in Chapter 2, Section 2.1. Staphylococcal species were presumptively and definitively identified as described in Chapter 2, Sections 2.1 and 2.8. Definitive species identification of isolates recovered on CBA plates incubated under aerobic or anaerobic conditions was determined by MALDI-TOF MS or Sanger-based sequencing of the 16S ribosomal RNA gene (184), as described in Chapter 2, Sections 2.8.3 and 2.8.4, respectively.

3.2.6. Whole-genome sequence analysis

Nucleic acid extraction and short-read WGS was performed as previously described in Chapter 2, Section 2.9. Short-read FASTQ files were assembled, quality assessed and subjected to whole-genome multilocus typing (wgMLST) and minimum spanning tree (MST) construction using BioNumerics software v8.0.

Short-read FASTQ files were also ssembled using the VELVET assembler within the Ridom SeqSphere+ software v7.0.4 program. Ridom SeqSphere+ was also used to identify secreted protein A gene (*spa*) types, antimicrobial resistance, and virulence genes from WGS datasets as previously described in Chapter 2, Section 2.9.5.

All WGS dataset files have been deposited in the NCBI Sequence Read Archive under BioProject PRJNA971107.

3.2.7. Statistical analysis

Statistical analyses including Mann Whitney, Kruskal Wallis and Wilcoxon Signed Rank tests based on related samples were performed using IBM SPSS Statistics v25 (IBM, Armonk, NY, USA). Significant results were indicated by P values < 0.05 and < 0.01.

3.3. Results

3.3.1. Types of ulcers sampled

All DFU debrided tissue samples were collected from 30 participants with type II diabetes attending the SJH foot clinic for routine debridement (Table 3.1) over three years. Samples from patients who participated multiple times were collected a minimum of two weeks apart. Of the 51 samples recovered, 24 were collected from patients with a prior LEA history and 13 were from patients with more than one active ulcer (range two – four ulcers) at the time of sample collection. The average age of each sampled ulcer was 32 weeks (median 13, range 0-500 weeks). The ulcers sampled were predominantly neuropathic (42/51 [82.4%]), neuroischaemic (9/51 [17.6%]) and diagnosed as Texas wound grades 1A (39/51, [76.5%]); superficial and lacking clinical signs of infection. Four ulcers exhibited clinical DFUI symptoms, which were graded as 1B, 1D and 3D. Four of the ulcers sampled had penetrated tendon, capsule, bone or joint tissue and were graded as 2A (*N*=2), 3C (*N*=1) and 3D (*N*=1).

3.3.2. Microbial load of tissues immersed in 1 ml volumes of saline or 200 ppm anolyte

The average (and median) bacterial loads recovered from 1 ml saline-treated tissues following aerobic and anaerobic culture on CBA and aerobic culture on Sa*Select*™ agar were $1.40\times10^6 \pm 5.28\times10^5$ (2.78×10^5), $9.23\times10^5 \pm 3.03\times10^5$ (5.00×10^5) and $6.29\times10^5 \pm 2.38\times10^5$ (3.20×10^5) CFU/g of debrided tissue, respectively (Table 3.2). In contrast, the corresponding average (and median) bacterial loads recovered from 1 ml anolyte-treated tissues following aerobic and anaerobic culture on CBA and aerobic culture on Sa*Select*™ agar were $5.11\times10^4 \pm 3.54\times10^4$ (8.05×10^3), $2.49\times10^4 \pm 9.14\times10^3$ (7.83×10^3) and $2.42\times10^4 \pm 1.48\times10^4$ (3.82×10^3) CFU/g, respectively. Significant reductions (Z -3.92, $P<0.01$) in the average bacterial load of 1 ml anolyte-treated tissues compared with l ml saline-treated tissues were observed following aerobic (2.0 log, 227.7-fold) and anaerobic (1.9 log, 74.7-fold) culture on CBA agar and aerobic culture (2.2 log, 1605.6 -fold) on Sa*Select*™ agar (Table 3.2 and Fig. 3.3).

Table. 3.2. Microbial loads of debrided ulcer tissues samples Ano1-Ano21 following immersion in 1 ml volumes of either saline or anolyte following incubation under aerobic, anaerobic and staphylococcal selective conditions

Sample	Tissue (mg)	Texas grade	Aerobic CBA[a] (CFU/g)				Anaerobic CBA[a] (CFU/g)				Aerobic Sa*Select*™ (CFU/g)			
			Saline	Anolyte	Fold red[b]	Log red	Saline	Anolyte	Fold red[b]	Log red	Saline	Anolyte	Fold red[b]	Log red
ANO1	25	1A	6.40E+04	2.00E+02	320.0	2.5	3.65E+04	2.25E+02	162.2	2.2	2.00E+04	1.00E-01	80.0	5.3
ANO2	35	1A	2.57E+03	1.00E-01	25714.3	4.4	4.90E+03	1.00E-01	4.7	4.7	2.80E+03	1.00E-01	28000.0	4.4
ANO3	35	1A	3.71E+06	9.78E+04	38.0	1.6	5.78E+06	1.44E+05	40.1	1.6	5.08E+04	2.80E+02	181.3	2.3
ANO4	30	1A	1.71E+06	1.93E+04	88.6	1.9	1.01E+06	2.00E+04	50.4	1.7	1.80E+06	2.70E+04	66.7	1.8
ANO5	35	3D	7.95E+06	7.15E+05	11.1	1.0	6.58E+05	3.30E+04	20.0	1.3	8.16E+04	3.50E+03	23.3	1.4
ANO6	35	1D	6.57E+04	2.03E+03	32.4	1.5	9.80E+05	1.60E+04	61.4	1.8	1.16E+06	2.98E+05	3.9	0.6
ANO7	35	1A	1.17E+05	4.76E+03	24.5	1.4	1.41E+05	6.16E+03	23.0	1.4	4.73E+06	3.85E+04	122.7	2.1
ANO8	35	1A	2.51E+05	6.90E+03	36.5	1.6	9.80E+04	9.24E+03	10.6	1.0	3.25E+05	1.09E+03	299.4	2.5
ANO9	35	1A	1.60E+06	6.72E+03	238.4	2.4	1.06E+05	1.82E+03	58.1	1.8	3.42E+05	8.51E+03	40.2	1.6
ANO10	20	1A	7.64E+06	4.96E+04	154.0	2.2	1.86E+06	6.24E+04	29.9	1.5	1.14E+06	6.20E+04	18.3	1.3
ANO11	25	1A	1.17E+06	9.20E+03	127.3	2.1	6.24E+05	8.45E+03	73.8	1.9	4.37E+05	9.20E+03	47.5	1.7
ANO12	35	1B	1.28E+06	4.34E+04	29.5	1.5	8.68E+05	3.78E+04	23.0	1.4	3.16E+05	1.02E+04	30.9	1.5
ANO13	35	1A	5.66E+05	6.30E+03	89.8	2.0	3.64E+05	3.85E+03	94.5	2.0	5.24E+05	4.13E+03	126.9	2.1
ANO14	35	1A	2.06E+05	2.42E+03	85.2	1.9	2.38E+05	9.10E+02	261.5	2.4	1.66E+05	9.80E+02	169.3	2.2
ANO15	30	3B	2.13E+05	1.22E+04	17.4	1.2	1.56E+05	4.44E+03	35.1	1.5	2.44E+05	6.84E+03	35.6	1.6
ANO16	25	1D	2.08E+05	1.25E+02	1664.0	3.2	4.51E+05	2.05E+03	220.0	2.3	4.48E+04	7.50E+01	596.7	2.8
ANO17	35	1A	3.04E+05	1.15E+04	26.5	1.4	5.49E+05	1.14E+04	48.1	1.7	2.04E+05	8.75E+02	233.6	2.4
ANO18	35	1A	1.39E+05	1.37E+04	10.2	1.0	1.01E+05	2.87E+03	35.1	1.5	1.67E+05	9.07E+03	18.5	1.3
ANO19	35	1A	5.21E+05	2.06E+04	25.3	1.4	2.86E+06	1.26E+05	22.7	1.4	4.62E+05	3.15E+03	146.7	2.2
ANO20	50	1A	2.30E+05	1.50E+02	1536.0	3.2	1.58E+06	7.20E+03	218.9	2.3	3.74E+05	2.00E+02	1870.0	3.3

Mean	**1.40E+06**	**5.11E+04**	**1513.4**	**2.0**	**9.23E+05**	**2.49E+04**	**74.7**	**1.9**	**6.29E+05**	**2.42E+04**	**1605.6**	**2.2**
SEM	**5.28E+05**	**3.54E+04**			**3.03E+05**	**9.14E+03**			**2.38E+05**	**1.48E+04**		
Median [c]	**2.78E+05**	**8.05E+03**	**61.6**	**1.8**	**5.00E+05**	**7.83E+03**	**44.1**	**1.7**	**3.20E+05**	**3.82E+03**	**101.4**	**2.1**

[a] Abbreviations: CBA; Columbia blood agar, CFU/g; Colony forming units per gram of tissue, Fold red; Fold reduction, Log red; Log reduction, SEM; Standard Error of the Mean

[b] A Mann-Whitney U test for independent samples indicated that the fold reduction distribution observed between samples treated in 1 ml volumes was significantly different *(P*<0.05)

[c] Wilcoxon Signed Rank Tests based on related samples deemed that the differences between the medians of saline- and anolyte-treated solutions was statistically significant (*P*<0.01) under all incubation conditions investigated.

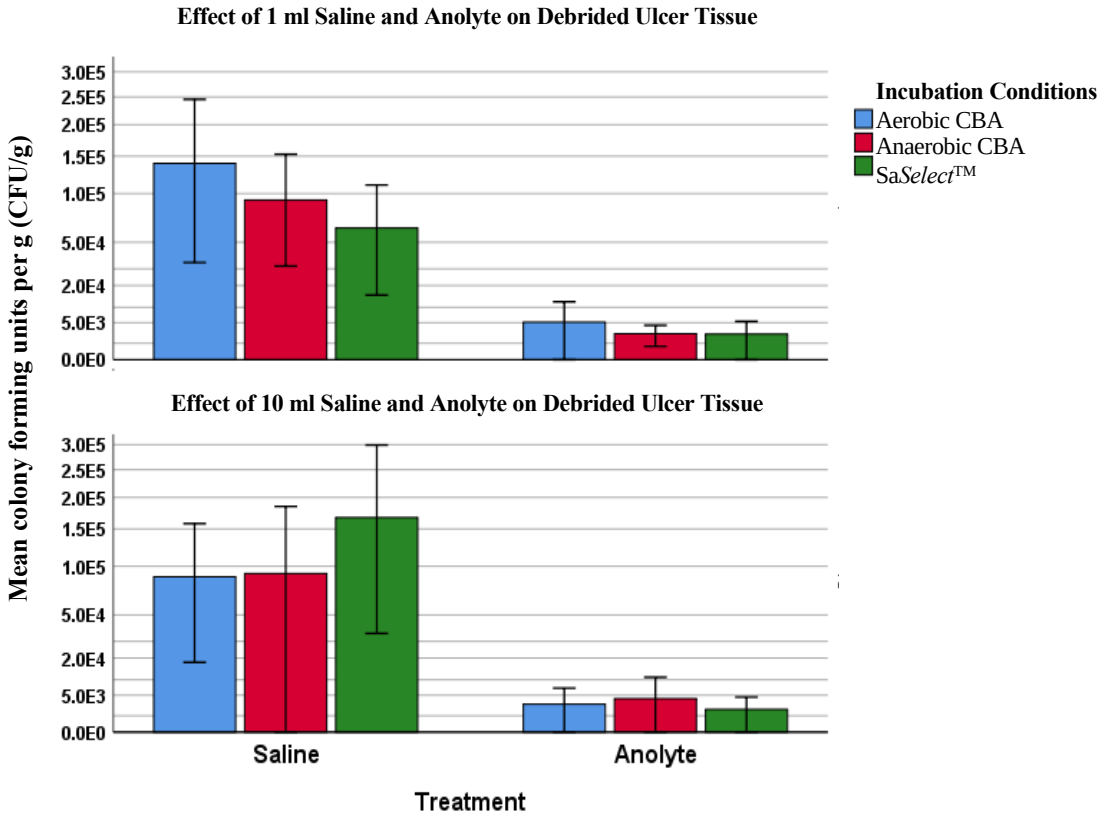


Figure 3.3. Mean microbial loads (CFU/g) of tissue in samples Ano1- Ano20 treated in 1 ml (Top Panel) and samples Ano21-Ano51 treated in 10 ml volumes (Bottom Panel) of sterile saline or 200 ppm anolyte followed by incubation under aerobic (blue), anaerobic (red) or aerobic Sa*Select*™ (green) incubation conditions. Error bars indicate standard error from the mean, Y axes indicate logarithmic scales. The mean bacterial loads of tissues following immersion treatment in 1 ml saline solution were 1.40×10^6, 9.23×10^5 and 6.29×10^5 CFU/g following aerobic and anaerobic culture on Columbia blood agar (CBA) or aerobic culture on Sa*Select*™, respectively. In contrast, the mean bacterial loads of tissues immersed in 1 ml of anolyte were significantly lower (P<0.01), 5.11×10^4, 2.49×10^4 and 2.42×10^4 CFU/g, following aerobic and anaerobic culture on CBA or aerobic culture on Sa*Select*™, respectively. Following immersion treatment in 10 ml saline solution, the mean bacterial loads of tissues were 8.78×10^5, 9.14×10^5 and 1.67×10^6 CFU/g following aerobic and anaerobic culture on CBA or aerobic culture on Sa*Select*™, respectively. In contrast, the mean bacterial loads of tissues immersed in 10 ml of anolyte were significantly lower (P<0.01), 2.86×10^4, 4.13×10^4 and 1.98×10^4 CFU/g following aerobic and anaerobic culture on CBA or aerobic culture on Sa*Select*™, respectively.

3.3.3. Microbial load of tissues immersed in 10 ml volumes of saline or 200 ppm anolyte

As previous studies have shown that anolyte FAC concentration decreases rapidly following contact with organic material (185). Experiments were undertaken to investigate if

increasing saline and anolyte immersion volumes further reduced the microbial load of anolyte-treated tissues. Thirty-one tissue samples were subjected to immersion-treatment in 10 ml volumes of saline or anolyte, respectively. Following 10 ml saline-treatment, the average (and median) bacterial loads recovered following aerobic and anaerobic culture on CBA and aerobic culture on Sa*Select*™ agar were $8.78\times10^5 \pm 3.50\times10^5$ (2.80×10^5), $9.14\times10^5 \pm 4.68\times10^5$ (4.16×10^5) and $1.67\times10^6 \pm 6.79\times10^5$ (2.97×10^5) CFU/g, respectively. In contrast, the average (and median) bacterial loads recovered from 10 ml anolyte-treated tissues following aerobic and anaerobic culture on CBA and aerobic culture on Sa*Select*™ agar were $2.86\times10^4 \pm 2.11\times10^4$ (2.30×10^3), $4.13\times10^4 \pm 3.43\times10^4$ (1.48×10^3) and $1.98\times10^4 \pm 1.33\times10^4$ (1.20×10^5) CFU/g, respectively (Table 3.3). Highly significant (Z-4.86, $P<0.01$) reductions in the average bacterial load (CFU/g) of 10 ml anolyte-treated samples compared to 10 ml saline-treated samples were observed following aerobic (2.2 log, 12,243.6-fold) and anaerobic (2.3 log, 2747.2-fold) culture on CBA, and aerobic culture (2.5 log, 9656.9-fold) on Sa*Select*™ agar (Table 3.3 and Fig. 3.3), respectively. Furthermore, the comparison of fold reductions observed between 1 ml and 10 ml treatment volumes revealed that, under anaerobic incubation conditions, the 10 ml treatment volume was significantly ($P<0.05$) more effective at reducing microbial bioburdens than the 1 ml treatment volume (Table 3.4, Fig. 3.4).

Table. 3.3. Microbial loads of debrided ulcer tissues samples Ano31- Ano51 following immersion in 10 ml volumes of either saline or anolyte following incubation in aerobic, anaerobic and staphylococcal selective conditions

Sample	Tissue (mg)	Texas grade	Aerobic CBA[a] (CFU/g)				Anaerobic CBA[a] (CFU/g)				Aerobic Sa*Select*™ (CFU/g)			
			Saline	Anolyte	Fold red[b]	Log red	Saline	Anolyte	Fold red[b]	Log red	Saline	Anolyte	Fold red[b]	Log red
ANO21	50	1A	3.46E+06	6.60E+05	5.2	0.7	1.48E+07	1.07E+06	13.8	1.1	7.70E+06	4.00E+05	19.3	1.3
ANO22	25	1A	7.68E+05	3.25E+02	2363.1	3.4	2.57E+05	1.03E+03	250.7	2.4	1.81E+05	3.75E+02	481.3	2.7
ANO23	35	1A	2.97E+05	3.19E+03	93.3	2.0	5.07E+05	7.70E+02	658.2	2.8	3.39E+05	1.05E+02	3226.7	3.5
ANO24	40	1A	2.63E+05	5.20E+02	505.8	2.7	4.16E+05	1.48E+03	281.1	2.4	2.68E+04	2.00E+02	134.0	2.1
ANO25	35	1A	3.28E+05	5.60E+02	585.7	2.8	3.50E+05	1.40E+02	2500.0	3.4	2.97E+05	4.20E+02	706.7	2.8
ANO26	40	1D	3.49E+05	8.80E+02	396.6	2.6	7.01E+05	4.40E+02	1592.7	3.2	3.73E+05	6.40E+02	582.5	2.8
ANO27	40	3A	4.74E+05	1.01E+04	46.8	1.7	6.94E+05	3.72E+03	186.7	2.3	7.20E+05	1.20E+03	600.0	2.8
ANO28	20	1A	1.60E+05	3.02E+03	53.0	1.7	1.39E+05	2.30E+03	60.5	1.8	5.24E+04	1.72E+03	30.5	1.5
ANO29	40	2A	4.05E+04	4.80E+02	84.4	1.9	5.00E+04	5.60E+02	89.3	2.0	2.32E+04	1.00E-01	232000.0	5.4
ANO30	25	1A	1.76E+05	1.23E+03	143.3	2.2	7.60E+04	1.08E+03	70.7	1.8	9.20E+04	1.10E+03	83.6	1.9
ANO31	40	1A	5.13E+05	4.03E+04	12.7	1.1	8.38E+05	4.22E+04	19.8	1.3	6.77E+05	3.71E+04	18.2	1.3
ANO32	35	1A	2.49E+05	7.00E+02	355.1	2.6	2.66E+05	3.50E+02	760.0	2.9	5.60E+04	4.20E+02	133.3	2.1
ANO33	45	2A	9.76E+05	3.20E+04	30.5	1.5	1.21E+06	2.74E+04	44.2	1.6	1.02E+06	7.38E+03	138.8	2.1
ANO34	45	1A	2.38E+06	8.73E+03	272.9	2.4	7.63E+05	3.42E+04	22.3	1.3	7.52E+05	1.78E+04	42.2	1.6
ANO35	45	1A	2.52E+05	3.56E+03	71.0	1.9	5.44E+05	4.55E+03	119.6	2.1	4.10E+04	4.50E+01	910.0	3.0
ANO36	45	1A	1.35E+06	3.78E+04	35.7	1.6	2.28E+05	1.03E+04	22.1	1.3	1.79E+07	6.30E+04	284.7	2.5
ANO37	45	1A	2.15E+05	2.30E+03	93.7	2.0	2.81E+05	3.33E+02	842.7	2.9	1.47E+06	1.00E+03	1472.0	3.2
ANO38	50	1A	2.56E+05	4.50E+03	56.9	1.8	5.40E+05	4.00E+03	135.0	2.1	3.12E+05	2.15E+03	145.1	2.2
ANO39	35	1A	2.64E+05	1.32E+04	20.1	1.3	2.13E+05	3.33E+03	64.0	1.8	2.16E+05	1.07E+04	20.2	1.3
ANO40	50	1A	1.46E+06	2.50E+03	583.2	2.8	2.03E+06	1.80E+03	1128.9	3.1	4.26E+06	1.23E+04	345.9	2.5

ANO41	42	1A	5.14E+04	9.24E+02	55.7	1.7	3.90E+04	8.82E+02	44.3	1.6	6.38E+04	8.20E+02	77.9	1.9
ANO42	10	1A	1.06E+07	1.82E+04	582.4	2.8	8.48E+05	2.02E+04	42.0	1.6	3.72E+06	6.60E+03	563.3	2.8
ANO43	35	1A	2.80E+05	2.69E+04	10.4	1.0	5.00E+05	4.56E+04	11.0	1.0	1.79E+05	1.09E+04	16.3	1.2
ANO44	35	1A	3.53E+05	1.40E+03	252.0	2.4	2.22E+05	9.45E+02	234.6	2.4	3.06E+05	2.94E+03	104.0	2.0
ANO45	45	1A	6.00E+04	1.89E+03	31.7	1.5	3.47E+04	2.30E+03	15.1	1.2	8.33E+04	2.16E+03	38.5	1.6
ANO46	45	1C	2.51E+05	8.10E+02	309.5	2.5	6.84E+05	2.70E+02	2533.3	3.4	2.29E+05	2.00E+03	114.5	2.1
ANO47	35	1A	4.47E+05	1.05E+02	4255.8	3.6	4.34E+05	1.40E+02	3100.0	3.5	2.61E+06	2.09E+02	12480.0	4.1
ANO48	34	1A	2.06E+03	6.80E+01	30.3	1.5	2.35E+03	3.40E+01	69.2	1.8	2.09E+04	3.40E+01	614.7	2.8
ANO49	50	1A	1.26E+04	1.00E+02	126.0	2.1	3.00E+03	1.00E-01	30000.0	4.5	4.32E+03	1.00E-01	43200.0	4.6
ANO50 [c]	50	1A	3.68E+04	1.00E-01	368000.0	5.6	4.00E+03	1.00E-01	40000.0	4.6	-	-	-	-
ANO51	45	1A	9.00E+05	1.01E+04	89.2	2.0	6.91E+05	2.75E+03	251.6	2.4	8.10E+06	1.04E+04	778.8	2.9
Average			**8.78E+05**	**2.86E+04**	**12243.6**	**2.2**	**9.14E+05**	**4.13E+04**	**2747.2**	**2.3**	**1.67E+06**	**1.98E+04**	**9656.9**	**2.5**
SEM			**3.50E+05**	**2.11E+04**			**4.68E+05**	**3.43E+04**			**6.79E+05**	**1.33E+04**		
Median [d]			**2.80E+05**	**2.30E+03**	**93.2944**	**2.0**	**4.16E+05**	**1.48E+03**	**135**	**2.1**	**2.97E+05**	**1.20E+03**	**145.116**	**2.16**

[a] Abbreviations: CBA; Columbia blood agar, CFU/g; Colony forming units per gram of tissue, Fold red; Fold reduction, Log red; Log reduction, SEM; Standard Error of the Mean

[b] A Mann-Whitney U test for independent samples indicated that the fold reduction distribution observed between samples treated in 1 ml volumes was significantly different ($P<0.05$)

[c] One of the saline-treated samples (Ano50) yielded no colonies following staphylococcal selective incubation and therefore this sample was excluded from the summation of results based on this set of incubation conditions only.

[d] Wilcoxon Signed Rank Tests based on related samples deemed that the differences between the medians of saline- and anolyte-treated solutions was statistically significant ($P<0.01$) under all incubation conditions investigated.

Table 3.4. Summary of microbial recovery (colony forming units/g (CFU/g)) and fold reduction from debrided foot ulcer tissue after immersion in 1 ml or 10 ml volumes of sterile saline or 200 ppm anolyte following incubation under aerobic, anaerobic and staphylococcal-selective conditions

Treatment volume (Samples)	**1 ml (Ano 1-20)**		**10 ml (Ano 21-51)**	
	Mean	**Median[a]**	**Mean**	**Median[a]**
Aerobic CBA[b] (CFU/g)				
Saline (CFU/g) [c]	1.40E+06	2.78E+05	8.78E+05	2.80E+05
Anolyte (CFU/g)	5.11E+04	8.1E+03	2.86E+04	2.30E+03
Fold reduction	1513.4	61.6	12243.6	93.3
Log reduction	2.0	1.8	2.2	2.0
Anaerobic CBA[b] (CFU/g)				
Saline (CFU/g) [c]	9.23E+05	5.00E+05	9.14E+05	4.16E+05
Anolyte (CFU/g)	2.49E+04	7.83E+03	4.13E+04	1.48E+03
Fold reduction[d]	74.7	44.1	2747.2	135
Log reduction	1.9	1.7	2.3	2.1
Aerobic Sa*Select*™ (CFU/g)				
Saline (CFU/g) [c]	6.29E+05	3.20E+05	1.67E+06	2.97E+05
Anolyte (CFU/g)	2.42E+04	3.82E+03	1.98E+04	1.20E+03
Fold reduction	1605.6	101.4	9656.9	145.1
Log reduction	2.2	2.1	2.5	2.2

[a] Wilcoxon Signed Rank Tests based on related samples deemed that the differences between the medians of saline- and anolyte-treated solutions was significant (P<0.01) under all incubation conditions and treatment volumes investigated.

[b] Abbreviations: CBA; Columbia blood agar, CFU/g; Colony forming units per gram of tissue, ppm; parts per million.

[c] Only 4/42 of the samples which yielded microbial loads >10^5 CFU/g, a threshold previously shown to impede wound healing (154), exhibited clinical signs of infection

[d] A Mann-Whitney U test for independent samples indicated that the fold reduction distribution observed between samples treated in 1- or 10 ml- treatment volumes was significantly different (P<0.05).

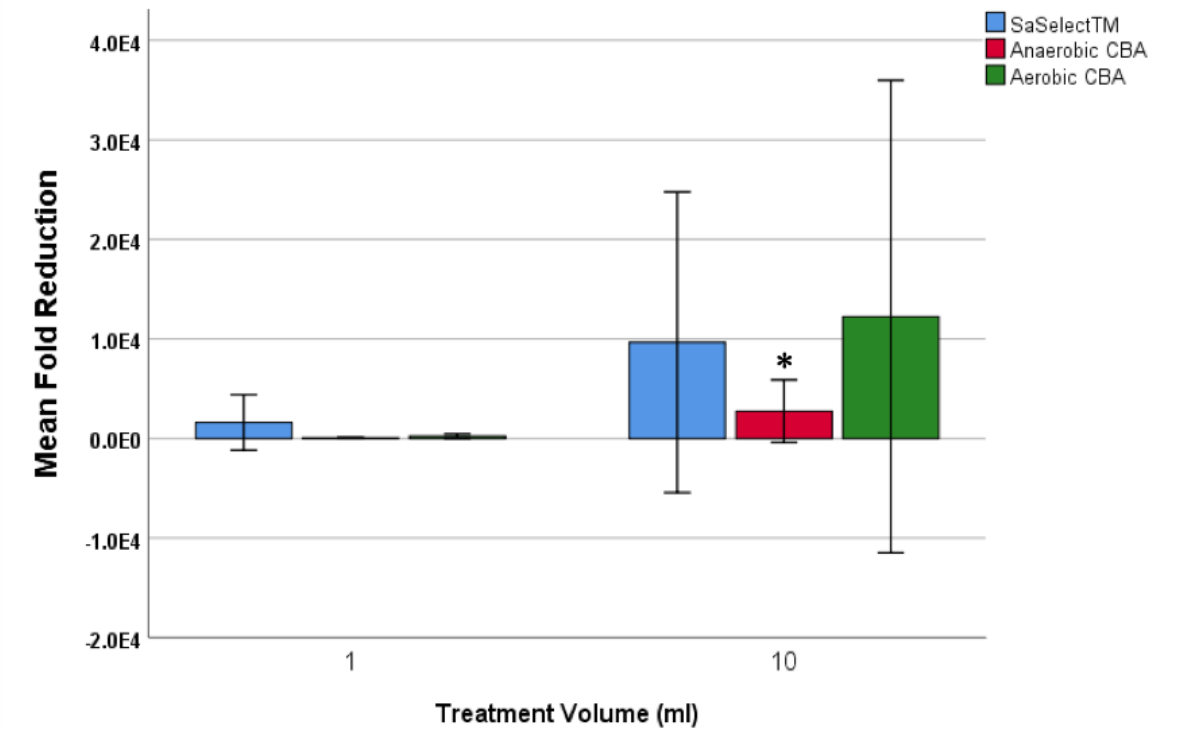


Figure 3.4. Mean fold reduction of microbial loads in samples treated in 1 ml (Ano1- Ano20) or 10 ml (Ano21-Ano51) volumes of 200 ppm freshly generated anolyte in comparison to treatment in equal volumes of sterile saline followed by incubation under aerobic Sa*Select*^{TM} (blue), anaerobic (red), or aerobic (green) incubation conditions. Error bars indicate standard error from the mean, Y axes indicate mean fold reductions by logarithmic scales. Abbreviations: CBA; Columbia blood agar. Statistical significance, determined as *P* values <0.05 is indicated by asterisks. A Mann-Whitney U test for independent samples indicated that the fold reduction distribution observed between anaerobically incubated samples treated in 1- or 10- ml treatment volumes was significantly different. Abbreviations: CBA; Columbia blood agar.

3.3.4. Bacterial isolates identified from debrided DFU tissue

Definitive species identification was performed on 237 isolates recovered from saline-and anolyte-treated tissues under aerobic and anaerobic culture conditions. These were selected to ensure a widespread representation of isolates remaining in each sample, under each incubation condition used and to ensure that there was no species-enrichment in anolyte-treated samples. *Staphylococcus* was the most common genus identified (44/51 [86.3%]), of which *S. aureus* was the most prevalent species recovered (30/51 [58.8%]; Table 3.5), followed by species belonging to the genera *Streptococcus* (9/51 [17.6%]), *Proteus* (7/51 [13.7%]), and *Enterococcus* (5/51 [9.8%]). Twelve patients participated in the study on multiple separate occasions, with at least two weeks between each sample collection. Distinct microbial populations were detected from these samples on each occasion, except for two samples collected from patient P4 in December 2022 and January 2023, both of which exclusively yielded *Proteus mirabilis* (Table 3.6).

Table 3.5. Numbers of isolates selected as representatives of each treatment and incubation type subjected to definitive species identification.

Incubation conditions	**Treatment type**	
	Saline (*N*)	**Anolyte (*N*)**
Aerobic CBA	56	43
Anaerobic CBA	64	74
Total	120	117

Abbreviations: CBA; Columbia blood agar.

Table 3.6. 16S rRNA sequencing data of representative isolates recovered from distinct patient tissue samples, and *S. aureus* STs identified from patients harbouring *S. aureus*

Sample	Patient	Species Identified (*N*)[a]	*S. aureus* STs
Ano1	P1	*Corynebacterium striatum* (2)	-
Ano2	P2	*Streptococcus agalactiae* (2)	-
Ano3	P3	*Streptococcus agalactiae* (2)	-
Ano4	P4	*Enterococcus faecalis* (4), *Staphylococcus aureus* (5)	ST30 (2)
Ano36	P4	*Proteus mirabilis* (8)	
Ano38	P4	*Proteus mirabilis* (4)	
Ano45	P4	*S. aureus* (4), *S. epidermidis* (2)	ST15 (1)
Ano5	P5	*Staphylococcus aureus* (3)	ST45 (1)
Ano6	P6	*Staphylococcus capitis* (1)	
Ano7	P7	*Staphylococcus aureus* (2)	ST1 (2)
Ano8	P8	*Streptococcus agalactiae* (2), *Staphylococcus simulans* (2)	
Ano10	P9	*Staphylococcus simulans* (1), *Staphylococcus aureus* (1)	ST39 (1)
Ano12	P10	*Enterobacter aerogenes* (1), *Prevotella buccalis* (2)	
Ano31	P10	*S. aureus* (4), *Enterococcus faecalis* (1)	ST5 (1)
Ano14	P11	*Staphylococcus capitis* (1), *Staphylococcus haemolyticus* (2)	
Ano15	P12	*S. aureus* (1)	ST2416 (1)
Ano16	P13	*S. epidermidis* (1), *Finegolida magna* (2)	
Ano17	P14	*Staphylococcus simulans* (1)	
Ano18	P15	*Staphylococcus simulans* (1)	
Ano19	P16	*Staphylococcus caprae* (1)	
Ano26	P16	*S. aureus* (3)	ST7 (2)
Ano20	P17	*Staphylococcus pseudintermedius* (4), *Staphylococcus pettenkofer*i (2)	
Ano28	P17	*Streptococcus dentisani* (1), *Sphingomonas paucimobilis* (1), *S. aureus* (6), *Streptococcus mitis* (1), *Haemophilis influenzae* (1)	ST5 (2)
Ano37	P17	*S. aureus* (4), *Staphylococcus epidermidis* (1)	ST5 (2)
Ano49	P17	*S. aureus* (2)	ST5 (2)
Ano21	P18	*S. aureus* (5), *Streptococcus agalactiae* (1)	ST5 (2)
Ano30	P18	*S. aureus* (6)	ST5 (2)
Ano48	P18	*Proteus mirabilis* (1), *Staphylococcus aureus* (1), *Streptococcus agalactiae* (1)	ST5 (1)
Ano13	P19	*S. aureus* (3)	ST22 (2)
Ano27	P19	*Staphylococcus aureus* (4), *Corynebacterium striatum* (2), *Enterococcus faecalis* (4), *Sphingomonas paucimobilis* (1), *Moraxella osloensis* (1)	ST1 (1)
Ano50	P19	*S. aureus* (2), *Proteus mirabilis* (1)	ST1 (2)
Ano9	P20	*S. epidermidis* (2), *Staphylococcus simulans* (1)	
Ano25	P20	*S. aureus* (7)	ST1 (2)
Ano41	P20	*Staphylococcus capitis* (2)	
Ano51	P20	*Proteus mirabilis* (1), *Escherichia coli* (3), *Staphylococcus aureus* (1)	ST1 (1)
Ano32	P21	*Escherichia coli* (2), *Klebsiella pneumoniae* (3)	

Ano34	P21	*Corynebacterium amycolatum* (1), *S. aureus* (3), *Enterobacter hormaechei* (1), *Enterobacter cloacae* (1), *Corynebacterium striatum* (1)	ST5 (2)
Ano11	P22	*Streptococcus agalactiae* (5)	
Ano22	P22	*Staphylococcus saprophyticus* (2), *Staphylococcus haemolyticus* (1)	
Ano43	P22	*Proteus mirabilis* (6)	
Ano39	P23	*Staphylococcus aureus* (1), S*taphylococcus simulans* (1), *Proteus mirabilis* (1), *Streptococcus agalactiae* (2)	ST5 (1)
Ano44	P23	*Staphylococcus ureilyticus* (2), *Proteus mirabilis* (2), *Staphylococcus caprae* (1)	
Ano24	P24	*Staphylococcus aureus* (6), *Esherichia coli* (3)	ST15 (2)
Ano40	P24	*Staphylococcus aureus* (3), *Staphylococcus warneri* (1), *Staphylococcus capitis* (1)	ST15 (2)
Ano33	P25	*Staphylococcus aureus* (3), *Staphylococcus ureilyticus* (1), *Enterococcous faecalis* (2)	ST15 (1), ST 398 (1)
Ano35	P26	*Staphylococcus aureus* (4), *Enterococcus faecalis* (4), *Alcalingenes faecalis* (1)	ST5 (1)
Ano29	P27	*Staphylococcus simulans* (1), *Sphingomonas paucimobilis* (4), *Staphylococcus aureus* (1)	ST30 (1)
Ano23	P28	*Staphylococcus aureus* (5)	ST27 (2)
Ano42	P29	*Staphylococcus aureus* (4), *Staphylococcus warneri* (1), *Staphylococcus haemolyticus* (1)	ST8347 (2)
Ano46	P29	*Staphylococcus aureus* (3), *Pseudomonas aeruginosa* (1), *Streptococcus oralis* (1), *Corynebacterium striatum* (1)	ST8347 (2)
Ano47	P30	*Staphylococcus aureus* (2), *Staphylococcus saprophyticus* (2)	ST5 (2)

[a] Species identification was performed on 237 isolates recovered from aerobically incubated saline-treated tissue cultures (56 isolates from 31 patients), aerobically incubated anolyte-treated tissue cultures (43 isolates from 24 patients), anaerobically incubated saline-treated tissue cultures (64 isolates from 35 patients), and anaerobically incubated anolyte-treated tissue cultures (74 isolates from 35 patients).

3.3.5. Whole genome sequencing of *S. aureus*

As *S. aureus* was the predominant organism recovered from 30/51 (58.8%) distinct tissue samples, 50 *S. aureus* isolates selected as representatives of each distinct tissue sample were investigated by WGS. All isolates were methicillin-susceptible *S. aureus* (MSSA) and lacked the *mecA/C* genes. Other antimicrobial resistance genes detected included *blaZ* (33/50 [66.0%]), *fosB* (34/50 [68.0%]), *erm*(A) (4/50 [8.0%]) and *qacC* (5/50 [10.0%]). Genes encoding pore-forming toxins were detected in all 50 isolates, including the *hla* alpha-haemolysin gene (44/50 [88.0%]) and the *luk*D/E leukocidin-encoding genes (35/50 [70%]). The Panton-Valentine Leukocidin *lukF/S-PV* genes, the *edin* epidermal cell differentiation inhibitor toxin gene, and exfoliative toxin-encoding genes *etA/B/D* were not detected in any of the 50 isolates investigated. Immune evasion cluster (IEC) and enterotoxin genes including *sea/g/h/i/k/m/n/o/q/u* were detected in 41/50 (82.0%) and 49/50 (98.0%) isolates, respectively. The *tst1* gene encoding the toxic shock syndrome toxin was identified in 3/50 (6.0%) isolates (Table 3.7).

3.3.6. Population analysis of *S. aureus* using whole-genome multilocus sequence typing (wgMLST)

Fifteen distinct *spa* types and 12 distinct sequence types (STs) were identified, of which ST5 (t067, t002, t1082) (*N*=18), ST1 (t127) (*N*=9), and ST15 (t084) (*N*=6) predominated (Table 3.7). Isolates belonging to these STs have previously been described in oro-nasal and ulcer sites of people with diabetes in Ireland (186). Of the seven patients from whom *S. aureus* was recovered on more than one occasion, persistent STs and *spa* types were identified in three patients (P20, P24, P29) and exhibited a maximum of four allelic differences (Fig. 3.5). Multiple ST5 isolates with distinct *spa* types were recovered from two patients <4 months apart (Table 3.7). The ST5-t002 strains originally recovered from Patient 17 were subsequently replaced by ST5-t067 strains (separated from t002 isolates by 270 wgMLST allelic differences). Patient 18 yielded ST5-t067, ST5-t1082 and ST5-t067 strains on the first, second and third samplings, respectively, indicative of persistent co-colonisation with multiple distinct strains (>396 wgMLST allelic differences). Strain replacement was also observed in two patients; P4 (ST30 to ST15) and P19 (ST22 to ST1) from whom isolates were recovered >24 months apart (Table 3.7).

Table 3.7. Whole-genome sequence-based investigation of *Staphylococcus aureus* isolates recovered.

Patient	Isolate name	Treatment	Incubation conditions	ST	*spa* type	AR genes	PVL	Capsule genes	VF Genes	IEC type[a]
P10	Ano31-1	Saline	Aerobic	ST5	t067	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sem, sen, seo, seu*	B
P12	Ano15-1	Saline	Aerobic	ST2416	t2293	*blaZ, fosB*	Neg	cap5J, K	*hla, sel*	D
P16	Ano26-2	Saline	Aerobic	ST7	t091	*blaZ*	Neg	cap8H, I, J, K	*hla*	G
P16	Ano26-3	Saline	Aerobic	ST7	t091	*blaZ*	Neg	cap8H, I, J, K	*hla*	G
P17	Ano28-5	Anolyte	Aerobic	ST5	t002	*erm*(A), *fosB*	Neg	cap5H, J, K	*hla, seg, sei, sen, seo, seu*	C
P17	Ano28-6	Anolyte	Aerobic	ST5	t002	*erm*(A), *fosB*	Neg	cap5H, J, K	*hla, seg, sei, sen, seo, seu*	C
P17	Ano37-1	Saline	Aerobic	ST5	t067	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sem, sen, seo, seu*	B
P17	Ano37-3	Anolyte	Aerobic	ST5	t067	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sem, sen, seo, seu*	B
P17	Ano49-3	Saline	Anaerobic	ST5	t067	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sem, sen, seo, seu*	B
P17	Ano49-4	Saline	Anaerobic	ST5	t067	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sem, sen, seo, seu*	B
P18	Ano21-1	Saline	Aerobic	ST5	t067	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sem, sen, seo, seu*	B
P18	Ano21-4	Anolyte	Aerobic	ST5	t067	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sem, sen, seo, seu*	B
P18	Ano30-1	Saline	Aerobic	ST5	t1082	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sen, seo, seu*	B
P18	Ano30-3	Anolyte	Aerobic	ST5	t1082	*blaZ, fosB, qacC*	Neg	cap5H, J, K	*hla, seg, sei, sen, seo, seu*	C
P18	Ano48-6	Saline	Anaerobic	ST5	t067	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sem, sen, seo, seu*	B
P19	Ano13-7	Saline	Anaerobic	ST22	t18906	*blaZ*	Neg	cap5H, J, K	*hla, sei, sem, sen, seo, seu*	-
P19	Ano13-8	Saline	Anaerobic	ST22	t18906	*blaZ*	Neg	cap5H, J, K	*hla, sei, sem, sen, seo, seu*	-
P19	Ano27-1	Saline	Aerobic	ST1	t127	*blaZ, fusC*	Neg	cap8I, J, K	*seh*	E
P19	Ano50-3	Saline	Anaerobic	ST1	t127	*blaZ, fusC*	Neg	cap8I, J, K	*seh*	E
P19	Ano50-4	Anolyte	Anaerobic	ST1	t127	*blaZ, fusC*	Neg	cap8I, J, K	*seh*	E
P20	Ano25-1	Saline	Aerobic	ST1	t127	*blaZ, fusC*	Neg	cap8I, J, K	*seh*	E
P20	Ano25-2	Saline	Aerobic	ST1	t127	*blaZ, fusC*	Neg	cap8I, J, K	*seh*	E

P20	Ano51-7	Anolyte	Anaerobic	ST1	t127	*fusC*	Neg	cap8I, J, K	*seh*	E
P21	Ano34-2	Saline	Aerobic	ST5	t067	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sen, seo, seu*	B
P21	Ano34-5	Anolyte	Aerobic	ST5	t067	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sen, seo, seu*	B
P23	Ano39-3	Anolyte	Aerobic	ST5	t067	*fosB*	Neg	cap5H, J, K	*hla, seg, sei, sem, sen, seo, seu*	B
P24	Ano24-1	Saline	Aerobic	ST15	t084	*blaZ, fosB*	Neg	cap8H, I, J, K	*hla*	C
P24	Ano24-4	Anolyte	Aerobic	ST15	t084	*blaZ, fosB*	Neg	cap8H, I, J, K	*hla*	C
P24	Ano40-1	Saline	Aerobic	ST15	t084	*blaZ, fosB*	Neg	cap8H, I, J, K	*hla*	C
P24	Ano40-4	Anolyte	Aerobic	ST15	t084	*blaZ*	Neg	cap8H, I, J, K	*hla*	C
P25	Ano33-1	Saline	Aerobic	ST15	t084	*blaZ, fosB*	Neg	cap8H, I, J, K	*hla*	C
P25	Ano33-6	Anolyte	Aerobic	ST398	t1451	*blaZ*	Neg	cap5H, J, K	*hla*	C
P26	Ano35-1	Saline	Aerobic	ST1	t127	*blaZ, fosB, qacC*	Neg	cap8I, J, K	*hla, she, sek, seq*	D
P26	Ano35-5	Anolyte	Aerobic	ST5	t1082	*blaZ, fosB, qacC*	Neg	cap5H, J, K	*hla, seg, sei, sem, sen, seo, seu*	C
P27	Ano29-9	Saline	Anaerobic	ST30	t012	*blaZ, fosB*	Neg	cap8H, I, J, K	*hla, sei, sem, sen, seo, tst1*	E
P28	Ano23-1	Saline	Aerobic	ST27	t100	*blaZ, fosB*	Neg	cap5H, J, K	*hla, seb, sei, sen, seo, seu*	B
P28	Ano23-2	Anolyte	Aerobic	ST27	t100	*blaZ, fosB*	Neg	cap5H, J, K	*hla, seb, sei, sen, seo, seu*	B
P29	Ano42-1	Saline	Aerobic	ST8347	t190	*blaZ, fosB*	Neg	cap5H, cap5K	*hla*	-
P29	Ano42-4	Anolyte	Aerobic	ST8347	t190	*blaZ, fosB*	Neg	cap5H, cap5K	*hla*	-
P29	Ano46-5	Saline	Anaerobic	ST8347	t190	*blaZ, fosB*	Neg	cap5H, cap5K	*hla*	-
P29	Ano46-7	Anolyte	Anaerobic	ST8347	t190	-	Neg	cap5H, cap5K	*hla*	-
P30	Ano47-2	Saline	Aerobic	ST5	t1082	*blaZ, fosB, qacC*	Neg	cap5H, J, K	*hla, seg, sei, sen, seo, seu*	C
P30	Ano47-4	Anolyte	Aerobic	ST5	t1082	*blaZ, fosB, qacC*	Neg	cap5H, J, K	*hla, seg, sei, sen, seo, seu*	C
P4	Ano4-14	Saline	Anaerobic	ST30	t363	*blaZ, erm*(A), *fosB*	Neg	cap8H, I, J, K	*hla, seb, sei, sen, seo, seu*	-
P4	Ano4-15	Saline	Anaerobic	ST30	t363	*blaZ, erm*(A), *fosB*	Neg	cap8H, I, J, K	*hla, seb, sei, sen, seo, seu*	-
P4	Ano45-2	Saline	Aerobic	ST15	-	*blaZ, fosB*	Neg	cap8H, I, J, K	*hla*	C
P5	Ano5-10	Anolyte	Anaerobic	ST45	t230	*blaZ*	Neg	cap8H, I, J, K	*hla, sec, seg, sei, sel, sen, seo, seu, tst1*	B

P7	Ano7-1	Saline	Aerobic	ST1	t127	*blaZ*	Neg	cap8H, I, J, K	*hla, seh, sek, seq*	E
P7	Ano7-2	Saline	Aerobic	ST1	t127	-	Neg	cap8H, I, J, K	*hla, seh, sek, seq*	E
P9	Ano10-8	Anolyte	Anaerobic	ST39	t3132	*blaZ, fosB*	Neg	cap8H, I, J, K	*hla, sel, seo, tst1*	-

Abbreviations; ST; Sequence Type, AR; antimicrobial resistance, VF; virulence factor, IEC; immune evasion cluster.

[a] Immune evasion cluster (IEC) types: *chp, scn, sak* (B), *chp, scn* (C), *scn, sak, sea* (D), *scn, sak* (E), *scn, sak, sep* (G).

A wgMLST-based MST revealed the presence of three distinct clusters of closely related isolates, within which neighbouring isolates were separated from each other by a maximum of three allelic differences (Fig. 3.5), considerably below the previously proposed ≤ 24 allelic difference thresholds for determination of isolate close relatedness (Schürch *et al.*, 2018). Cluster 1 comprised ST5, t067 isolates (*N*=11) from five patients, Cluster 2 comprised ST5, t1082 isolates (*N*=5) from three patients and Cluster 3 comprised ST1, t127 isolates (*N*=6) from two patients. All the isolates within these three clusters were recovered within the same four-month period. Four isolates recovered from Patient P24 were separated from each other by a maximum of two allelic differences. One isolate recovered from P4 (Ano45-2) was separated from the most closely related isolate (Ano24-1) from P24 by only two allelic differences (Fig. 3.5). These findings indicated transmission of specific strains of *S. aureus* among some of the study patients.

3.3.7. Association of microbial loads with clinical ulcer features

Four ulcers exhibited clinical signs of infection and yielded $\geq 10^5$ CFU/g, a microbial threshold associated with impeded wound healing (154); however, an additional 38/51 (74.5%) saline-treated samples also yielded $\geq 10^5$ CFU/g (Table 3.8), despite lacking clinical signs of infection. The prevalence of *S. aureus* and microbial load distribution of saline-treated samples incubated under each of the three culture conditions was compared based on the following clinical criteria: clinical signs of infection, neuropathic or neuroischaemic ulcers, distinct Texas classifications, number of active ulcers, age of oldest ulcer and prior LEA history. Of these criteria, the prevalence of *S. aureus* and the average and median microbial load in anaerobically incubated saline-treated samples was significantly higher ($P<0.05$) in patients with LEA history (Table 3.8).

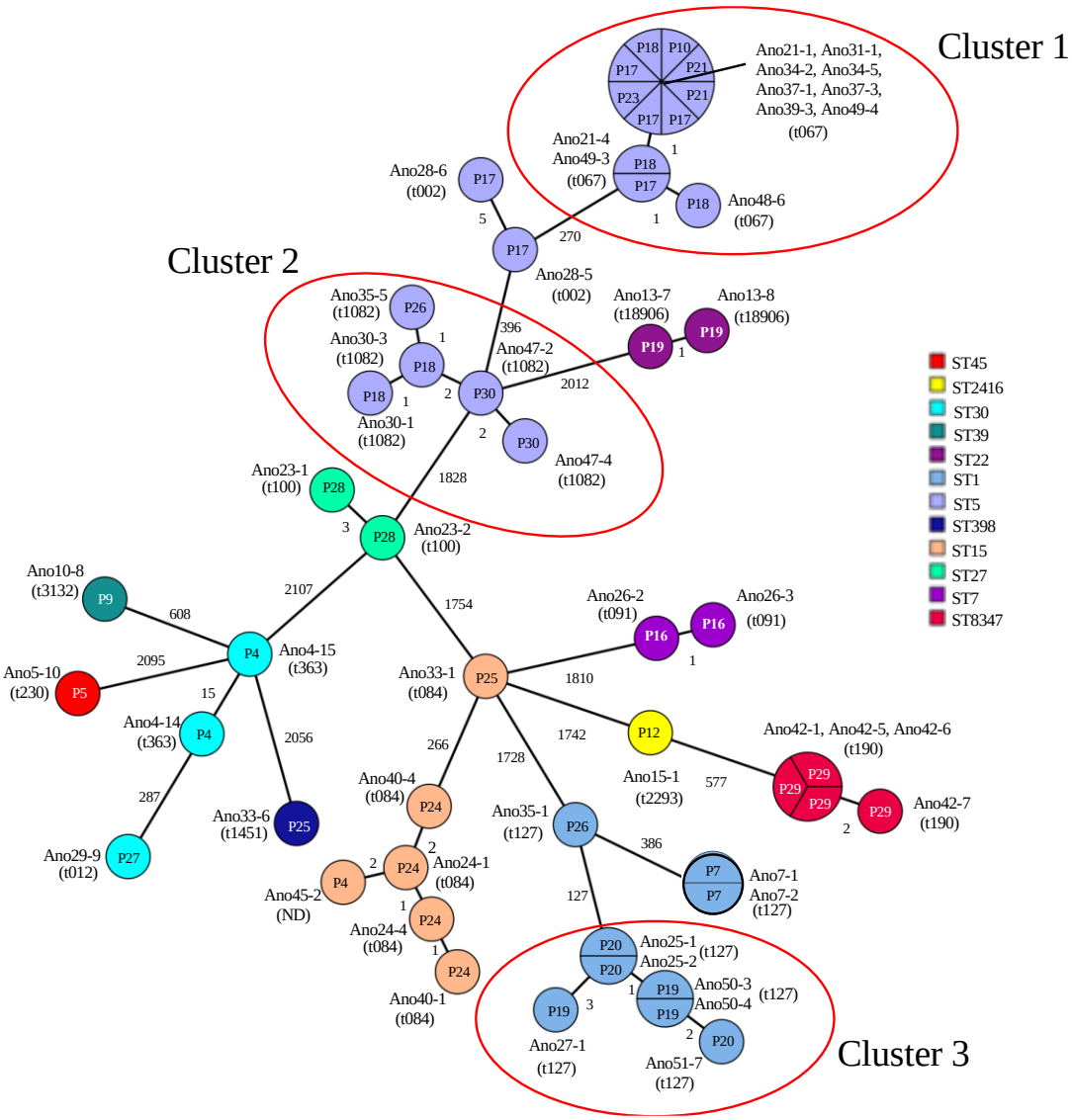


Figure 3.5. Minimum spanning tree based on wgMLST of 50 *S. aureus* isolates from 30 debrided tissue samples from 20 patients. Distinct patients are indicated by corresponding identifiers within each node. Node colours indicate 12 distinct sequence types (STs) according to the legend. Labels beside each node indicate isolate names and *spa* types. Integers above branches indicate the number of allelic differences between neighbouring isolates. Three distinct clusters (encircled in red) are apparent; Cluster 1 comprises ST5, t067 isolates recovered from five separate patients (P10, P17, P18, P21, P23) over the same four-month period in which neighbouring isolates are separated by a maximum of one allelic difference. Cluster 2 comprises ST5, t1082 isolates recovered from three distinct patients (P18, P26 and P30) over the same one-month period in which neighbouring isolates are separated by a maximum of two allelic differences. Cluster 3 comprises ST1, t127 isolates recovered from two separate patients (P19 and P20) over a four-month period within which neighbouring isolates are separated by a maximum of three allelic differences.

Table 3.8. Correlation of microbial loads and *S. aureus* prevalence with clinical ulcer features.

Ulcer features	Incubation conditions			Statistical test
	Aerobic	Anaerobic	Staphylococcal Selective	
Distribution of saline-treated microbial loads with clinical ulcer features following incubation in three distinct conditions				
Ulcer type (Texas)	NS	NS	NS	Mann Whitney
Neuropathic/Neuroischemic	NS	NS	NS	Mann Whitney
Amputation history	NS	0.04**	NS	Mann Whitney
Ulcer age (weeks)	NS	NS	NS	Kruskal Wallis test
Number of active ulcers	NS	NS	NS	Kruskal Wallis test
Clinical indicators of infection	NS	NS	NS	Mann Whitney
Distribution of average log reduction with clinical ulcer features				
Ulcer type (Texas)	NS	NS	NS	Mann Whitney
Neuropathic/Neuroischemic	NS	NS	NS	Mann Whitney
Amputation history	NS	NS	NS	Mann Whitney
Ulcer age (weeks)	NS	NS	NS	Kruskal Wallis test
Number of active ulcers	NS	NS	NS	Kruskal Wallis test
Clinical indicators of infection	NS	NS	NS	Mann Whitney
Association of *S. aureus* with clinical ulcer features				
Ulcer type (Texas)		NS		Pearson Chi Square
Neuropathic/Neuroischemic		NS		Pearson Chi Square
Amputation history		0.04**		Pearson Chi Square
Ulcer age (weeks)		NS		Pearson Chi Square
Number of active ulcers		NS		Pearson Chi Square
Clinical indicators of infection		NS		Pearson Chi Square

Abbreviations, NS; Not significant

3.4. Discussion

A simple, inexpensive and highly effective novel DFUI treatment could potentially reduce DFUI-associated consequences considerably. Dublin Dental University Hospital water networks have been successfully maintained to an extremely high microbiological standard by residual treatment with 2 ppm anolyte for almost two decades (159, 185). Furthermore, research from this institution has demonstrated the safety of ECA solutions and their effectiveness at significantly reducing biofilm formation in dental unit waterlines, washbasin U-bend traps and wastewater pipes (159, 166, 185, 187, 188). Many previous studies reported the superiority of ECA solutions for wound disinfection and healing (169-172, 189-191), however, these studies were predominantly clinically-based, comparing healing outcomes between patients treated with ECA- or placebo- solutions. Variability including clinical criteria for defining DFUIs, antibiotic usage, patient-specific immune responses and healing abilities, likely confound the results of these studies. In addition, the solution generation conditions, composition, pH, storage times and conditions and the exact equipment involved in making the activated solution is lacking in many other publications makes it difficult to assess or compare efficacy.

Although relatively small, our study demonstrated significant microbial load reductions in debrided DFU tissue following immersion in 200 ppm pH 7.0 anolyte. As the present study investigated the microbial load reduction of *ex-vivo* debrided tissues, patient-specific immunity, treatments and healing capabilities did not influence the study outcome. Significant microbial bioburden reductions were observed in anolyte-treated tissues using both 1 ml and 10 ml treatment volumes relative to saline under all culture conditions. However, increasing the immersion volumes from 1 ml to 10 ml further significantly reduced microbial loads recovered from debrided samples ($P<0.05$) under anaerobic incubation conditions. As the FAC in anolyte decreases rapidly upon contact with organic material, this finding highlights the importance of supplying excess anolyte-treatment volumes. The improved performance observed with the larger ratio of anolyte solution to tissue is in keeping with the understanding that more activation energy and reactants will be available to act, suggesting that an irrigation stream may be the ideal mode to apply the solution. Increasing the strength of anolyte for further improved performance in treating DFUIs is also an option. In 2020 an EU Biocidal Products Committee published a risk assessment and opinion noting no cause for concern for topical skin use of anolyte at 300 ppm FAC (192).

Carefully designed randomised control trials will be necessary to further explore the efficacy of anolyte immersion on improved DFU healing outcomes.

Our study is limited by the fact that 39/51 (76.5%) of the ulcers sampled were Type 1A; superficial ulcers lacking clinical signs of infection. Diagnosis of DFUIs is often based on subjective identification of typical infection symptoms (181), which are less clearly defined in those with immune dysfunction, neuropathy or ischaemia. Our study revealed that only 4/42 (9.5%) DFUs which yielded $>10^5$ CFU/g tissue exhibited clinical symptoms of infection indicating that DFUIs are very likely underdiagnosed. The association of *S. aureus* and higher anaerobic microbial loads (Table 3.2) amongst patients with a prior LEA history is attributable to the presence of chronic and biofilm-infected DFUs that are more difficult to manage and therefore more likely to be associated with LEA. Anolyte has previously been shown to be a non-toxic and an effective microbiocidal agent capable of penetrating biofilms (185, 187) further supporting its suitability for chronic wound treatment.

Species identification was performed on isolates selected from each sample and a selection of treatment types and incubation conditions to carry out a limited survey of the range of organisms present. Interestingly, species such as *Streptococcus agalactiae*, *Finegoldia magna*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Prevotella buccalis* previously recovered from the oral cavity or gastrointestinal tract (193-197) were recovered from 17/51 (33.3%) DFU samples collected. Of the 12 patients who were sampled on more than one occasion, only one patient (P4) yielded an identical monomicrobial culture composed of *P. mirabilis* on two separate occasions (Table 3.6). Distinct microbial populations were revealed on separate occasions from the other 11 individuals, indicating that DFU microbial populations fluctuate.

Unsurprisingly, *S. aureus* was the predominant species recovered (30/51 [58.8%] ulcer samples) correlating with previous investigations, however in contrast to these studies, no MRSA were detected (198). The *S. aureus pvl* gene often associated with skin and soft tissue infections, was absent (Table 3.7), correlating with previous studies of DFU-based *S. aureus* populations (199). The low prevalence of antibiotic resistance genes reflected a relatively diverse MSSA population, likely correlating with the superficial nature and absence of biofilm of the wounds sampled. Sequence type replacement occured in two patients (P4 and P19) over two years. In two patients (P17 and P18), multiple ST1 isolates recovered over four months had distinct *spa* types (P17; t002 and t067, P18; t067 and t1082), whereas persistent STs and *spa* types were identified in three patients (P20, P24, P29) over a four-month period (Table 3.7). In each of these three patients, isolates from individual

patients recovered on separate occasions were identified as the same ST, *spa* type and exhibited a maximum of four allelic differences (Fig. 3.5). Strain type persistence amongst patients was complicated by the identification of two distinct ST5 clusters (Clusters 1 and 2; five isolates from five patients per cluster) and a ST1 cluster (Cluster 3; two isolates from two patients) of closely related isolates in each case. Isolates within clusters were very closely related (range 0-3 allelic differences) suggesting isolate transmission between patients (Fig. 3.5). It is possible that the release of fine particulate matter in clinical treatment areas following debridement procedures, or the congregation of patients with open wounds in clinical waiting areas may have led to microbial transmission between patients and warrants further investigation.

Routine DFUI treatment typically includes administration of oral and intravenous antibiotics for superficial and invasive infections, respectively. These treatments can be problematic in patients who have ischaemia, vascular disease or kidney disease, all of which are very common diabetes-related complications. Our study revealed that 200 ppm anolyte immersion is highly effective for debrided DFU tissue disinfection ($P<0.01$), resulting in an average 99.0% (2 log) microbial load reduction in comparison to saline-treated tissues. The reduction of average and median microbial loads of anolyte-treated tissues to $<10^5$ CFU/g indicates that the microbial burdens were reduced to levels that would not impede wound healing (154). Furthermore, anolyte safety has been previously demonstrated using reconstituted human oral epithelial models (185).

In conclusion, regular anolyte based DFU immersion has excellent potential to provide a non-toxic, inexpensive and highly effective means of DFUI prevention and treatment, in combination with conventional wound treatments such as debridement, dressing and offloading.

Chapter 4

Comparative Prevalence and Abundance of Staphylococci from Multiple Anatomical Sites of Patients with Type II Diabetes With and Without Diabetic Foot Ulcers

4.1. Introduction

4.1.1. Oral healthcare for people with diabetes in Ireland

Over the last century, diabetes has become one of the leading global health emergencies in both Eastern and Western countries, linked with a predisposition to many co-morbidities including cardiovascular disease, kidney disease, peripheral neuropathy, blindness and lower extremity amputation (200-202). Persistently poor glycaemic control is associated with the onset and progression of these diabetes-related complications.

Studies have shown that people with diabetes are more likely to have poor oral health and suffer from oral complications and associated diseases, such as extensive xerostomia (a reduction or complete absence of saliva), dental caries, oral candidiasis, lichen planus (a chronic inflammatory condition that affects the oral mucosal membranes), burning mouth syndrome and tooth loss (203-206). Despite the growing evidence linking oral health and diabetes management, oral health in Ireland is rarely considered by medical professionals during consultation with patients with diabetes (207). This omission represents a significant gap in the educational literature for diabetes management. Socioeconomic factors including income, education and access to healthcare play a huge role in diabetes management and burden. Previous research demonstrated that individuals with poorer socioeconomic status and education histories were more likely to develop diabetes, experience more complications and have an increased mortality rate than those of higher socioeconomic status (208, 209). These socioeconomic factors can result in healthcare fatigue or ‘diabetes burnout’, where individuals with diabetes become overwhelmed with the management of their condition. This can lead to reduced self-care and emotional well-being (210, 211). Furthermore, dental visits may be perceived overly expensive, anxiety-inducing or stressful for individuals with diabetes, and they may perceive dental procedures as stressors that may exacerbate their overall health or diabetes management (212). Thus, it is essential to inform patients and practitioners of the medical relevance of the bi-directional relationship between oral health and diabetes and to encourage people with diabetes to attend their dentist regularly and undertake oral hygiene routinely.

4.1.2. Periodontal diseases in people with diabetes

Periodontal disease (PD) is a plaque-associated microbially induced condition resulting in chronic inflammation of the periodontium. It is the most prevalent oral complication associated with diabetes and is often considered as the sixth diabetes-associated

complication, including cardiovascular disease, kidney disease, peripheral neuropathy, blindness and lower extremity amputation (203). The risk of PD development is between two- and three-fold higher in patients with diabetes due to hyperglycaemia of host tissues and reduced salivary flow (213). The molecular mechanisms that link PD with diabetes are not yet fully understood, however, the link appears to involve aspects of the inflammatory response, immune function and neutrophil activity, as well as cytokine release (214).

Gingivitis and periodontitis can also correlate directly with poor glycaemic control, as virulent invasive microbes may induce inflammatory markers that are associated with insulin resistance (215, 216). Individuals with poorly controlled diabetes (HbA1c > 9%) have been reported to experience severe PD more frequently than individuals without diabetes (217). Numerous studies have investigated the effectiveness of PD treatment in glycaemic control improvement in patients with diabetes, and many of these studies have been systematically reviewed (218-222). A consistent finding was that periodontal treatment is associated with reductions of HbA1c of 0.4%. Calabrese *et al.* conducted a study with 225 patients with diabetes who underwent periodontal treatment, all of whom demonstrated improved glycaemic control at the study endpoint (223). Bukleta *et al.* reported a significant HbA1c improvement in patients with type II diabetes following tooth extraction and periodontal cleaning, in comparison to non-diabetic patients who underwent the same treatment (224).

Chattopadhyay *et al.* concluded that oral bacteria may influence systemic diseases such as diabetes through release of pro-inflammatory cytokines (225), whereas Silva *et al.* reported that diabetes was associated with significant changes in the subgingival and salivary microbiome (226). The effects of diabetes on the oral microbiome and its association with periodontitis in mice was investigated by Xiao *et al.* who reported that, compared to control mice populations without diabetes, diabetes reduced the total diversity of the oral microbiome, but increased the levels of Proteobacteria (*Enterobacteriaceae*) and Firmicutes (*Enterococcus*, *Staphylococcus* and *Aerococcus*). Furthermore, by transferring the oral microbiota from both non-diabetic control mice and diabetic mice to germ-free recipient mice, greater periodontal inflammation and bone loss were observed in mice that received oral microbiota samples from diabetic mice (227).

The formation of periodontal pockets and tooth loss provide easy access for oral bacteria to the systemic circulatory systems (228). Periodontal pathogens, including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* have been detected in different organs and systems in the human body by

molecular DNA typing techniques (229, 230). Routine daily oral hygiene-related activities can provoke bacteraemia (231); for example, research suggests that biofilm accumulation and inflammation of the gingiva increased the incidence of bacteraemia after toothbrushing (232). It has been reported that toothbrushing is a common source of bacteraemia and a potential source of bacterial endocarditis (233, 234). Forner *et al.* measured the incidence of bacteraemia between periodontally healthy patients and patients suffering from PD following eating, toothbrushing and tooth scaling. None of the 60 subjects were detectably bacteraemic before any of the experimental procedures. Following chewing, four (20.0%) of the PD patients but none of the periodontally healthy individuals were bacteraemic. Following toothbrushing, two (10.0%) patients with PD but none of the periodontally healthy subjects were bacteraemic. Bacteraemia following scaling was detected in two (10.0%) periodontally healthy patients, in four (20.0%) gingivitis patients, and in 15 (75.0%) PD patients (235).

4.1.3. Staphylococci in the oro-nasal cavity of people with periodontal disease

Previous research reported that staphylococcal populations are more prevalent in the oral cavities of patients with PD than in individuals without PD (236). O'Connor *et al.* (2018) reported that *S. epidermidis* was significantly more prevalent in the periodontal pockets and subgingival sites of patients with PD in contrast to orally healthy participants ($P<0.05$). This report suggested that the increased prevalence of oral *S. epidermidis* in patients with PD likely results in an increased abundance of *S. epidermidis* in subgingival sites and periodontal pockets. In contrast, other previous research reported that the most prevalent species in the oral cavity of patients with PD was *S. aureus* (237). O'Connor *et al.* (2018) reported that *S. aureus* was almost four times less prevalent and 19 times less abundant than *S. epidermidis* in the oral cavities of patients with PD, possibly due to increased competition between staphylococcal species within oral biofilms (236). Despite not being considered as significant periodontal pathogens, increased oral carriage of staphylococci, particularly *S. aureus* and *S. epidermidis* could have implications for systemic diseases in patients with PD. O'Connor *et al.* (2018) also investigated two or more isolates of *S. aureus* from separate sample sites per patient from 18 participants with PD, and reported that 17/18 (94.4%) participants harboured isolates in distinct anatomical sites that belonged to the same clonal complexes (CCs). It was concluded that trafficking likely occurred between oral, nasal, and subgingival sites (236). The same investigation also analysed two or more isolates of *S. epidermidis* recovered from multiple, distinct anatomical sites of 17, 20 and 25 patients with

PD, healthy patients with oral implants and orally healthy controls using DNA microarray profiling, respectively. Trafficking of *S. epidermidis* was inferred based on the similarity of virulence and resistance gene profiles in the *S. epidermidis* population. Presumptive *S. epidermidis* trafficking was detected in 11/62 (17.7%) of participants (236). These findings suggest that the oro-nasal cavity is likely an endogenous staphylococcal reservoir in patients with PD, and that these populations likely migrate between oral and nasal sites frequently.

4.1.4. Staphylococcal species and diabetic foot ulcer infection

Diabetic foot ulcer infections are a common and potentially disastrous complication associated with DFUs, affecting approximately 60% of DFU patients. *Staphylococcus aureus* is the predominant causative agent of DFUIs, associated with either deep or superficial infections (238-242) however, many other types of bacteria, including other staphylococcal species are also highly prevalent. Coagulase-negative staphylococci, *Micrococcus* spp., *Bacillus* spp. and C*orynebacterium spp.*, which are part of the normal skin flora, have been frequently isolated from DFUs, although they are not usually considered pathogenic unless isolated from deep wounds (243). Many previous publications have described the consistent recovery of CoNS from DFUs, however these CoNS were very rarely identified to the species level. González *et al.* reported that the most common microbial species isolated from DFUs were staphylococci: MSSA (26.9%); MRSA (5.3%); and CoNS (17.2%), however no definitive species identification of CoNS species recovered was performed (244). Furthermore, Li *et al.* reported the microbiological characteristics of DFU patients in Beijing, China, and reported that the most prevalent organisms recovered were *Enterobacteriaceae* (41%) and *Staphylococcus* spp. (25.4%). The prevalence of *S. aureus* was reported to be 17.1%, with no detail of other staphylococcal species recovered (245). If osteomyelitis is suspected in patients with DFUs, a full bacteriological analysis is required to allow prescription of the most appropriate regimen of antibiotics (246). Loïez *et al.* reported the CoNS species *S. pettenkoferi* as the causative agent of DFU-associated osteomyelitis in a 63-year old patient using 16S PCR techniques (247).

There is a direct correlation between individuals who are persistent carriers of *S. aureus* and increased risk of *S. aureus*-associated infection (248). Nasal carriage of *S. aureus* is a major risk factor for community-acquired or nosocomial infections with *S. aureus* (249-251) and is also a potential risk factor for DFUs and DFUIs in patients (186, 252-254). Many other studies revealed associations between nasal and DFU carriage of *S. aureus* and MRSA using DNA microarray analysis, genotyping, and multilocus sequence typing (186, 252-

256). Translocation of pathogenic organisms has previously been reported, where an individual may unknowingly transfer a pathogen present on the hands to other anatomical sites, resulting in infection (257). As *S. aureus* is carried predominantly in the anterior nares and the oral cavity, the latter of which is commonly overlooked (258, 259). Self -inoculation for infection has been documented in individuals who are frequently exposed to potential *S. aureus* carriers in both the community and health care settings, indicating a potential risk factor for DFUIs (260, 261).

Previous research reported that staphylococci are also highly prevalent in the oro-nasal cavities of patients with type II diabetes (186). Out of 18 patients with type II diabetes, *S. epidermidis* was the predominant staphylococcal species detected in the oro-nasal cavity, recovered from 17/18 patients. *Staphylococcus aureus* was the second most frequently recovered staphylococcal species, detected in the oro-nasal cavity of 10 patients and was significantly more prevalent in patients with DFUs compared to patients without DFUs. *Staphylococcus aureus* was recovered from the ulcers of 7/13 (53.8%) patients. Of these seven patients, six also harboured *S. aureus* in the oro-nasal cavity. Whole genome sequencing analysis provided evidence that *S. aureus* isolates recovered from oro-nasal and ulcer sites of these six patients were genetically indistinguishable, providing highly supporting evidence for the research hypothesis that the oro-nasal cavity is a potential reservoir for DFUIs caused by staphylococci (186).

4.1.6. Study aims

Oral staphylococci have been shown to be more abundant and prevalent in patients with PD (236). Due to the bidirectional relationship between periodontal disease and diabetes, and the risk of DFUI development, it is important to evaluate the prevalence and abundance of oro-nasal staphylococci in patients with type II diabetes. Several studies have presented evidence that oro-nasal carriage of *S. aureus* is a potential risk factor for DFUIs (186, 249, 250, 253, 262, 263). Previous pilot research from this laboratory was limited in terms of patient numbers, however it provided highly supportive evidence of a link between oro-nasal and ulcer sites (186). This present study sought to conduct a more comprehensive investigation into the prevalence and abundance of staphylococci in multiple anatomical sites in people with diabetes with and without DFUs and to comparatively investigate clinical and microbiologically associated differences between the two cohorts of patients.

4.2. Materials and Methods

4.2.1. Ethical approval and patient recruitment

Consenting patients with type II diabetes undergoing routine DFU debridement at the St. James's Hospital Dublin (SJH) foot clinic between January 2020 and January 2023 were invited to participate in the study (Appendix 2). Suitable participants had type II diabetes, were over 18 years of age, capable of providing informed consent, had no underlying heart conditions such as cardiovascular disease and other serious underlying conditions apart from type II diabetes. Informed consent was obtained from patients recruited to the study by the Dental Hygienist, Podiatrist or Principal Investigator. Ethical approval (Approval No. 2019-12) was granted by the SJH and Tallaght University Hospital Joint Research Ethics Committee (Fig. 2.1).

4.2.2. Clinical assessment and sample collection

4.2.2.1. Periodontal assessment and oro-nasal, finger and toe sample collection

A comprehensive periodontal examination was performed on each participant by a qualified dental hygienist using a portable dental chair (Fig. 4.1). In the absence of dental hygienist availability, the periodontal assessment and periodontal sample collection was omitted from the relevant participants for the sampling session.

A full periodontal examination was completed using a North Carolina periodontal probe (BF Mulholland Ltd., Co. Antrim, UK). The presence/absence of each tooth, presence/absence and type of oral prostheses, clinical attachment loss, bleeding on probing, suppuration, plaque indexes according to the Silness- Loe index (https://capp.mau.se/methods-and-indices/) and pocket depth measurements were all recorded. The two deepest periodontal pockets were selected for sampling by the dental hygienist by gently probing the gums and periodontal tissues. Saliva and supra-gingival plaque were removed from these selected sampling sites using a TePe compact tuft brush (TePe Oral Hygiene Products Ltd., Somerset, UK). Each pocket selected for sampling was sampled using two sterile PerioPaper™ gingival fluid collection strips (Oraflow, NY, USA), each of which was placed and then removed using an angled UniDent College Tweezers (BF Mulholland Ltd. Glenavy Rd, Crumlin, United Kingdom), the first of which was be left *in situ* for 60 s, removed and placed in a sterile 2 ml micro screw-capped tube (Sarstedt) containing 1 ml tryptone soya broth (Oxoid, ThermoFischer Scientific, Hampshire, UK).

The second PerioPaper™ strip was then inserted into the same pocket site, left *in situ* for 30 s, removed and inserted into the same collection tube as the first PerioPaper™ strip.

The oral cavity was sampled using an oral rinse, which was obtained by providing each participant with a sterile 100 ml plastic container (Sarstedt AG & Co.) containing 25 ml sterile water. Each participant was instructed to rinse the oral cavity for 30 s with the water before returning the washings to the same container.

The anterior nares, skin of index finger and skin of the large toe (or a different toe if necessary) were all swabbed separately by the dental hygienist using separate nitrogen-gassed VI-packed sterile transport swabs (Sarstedt AG & Co.) for each site.

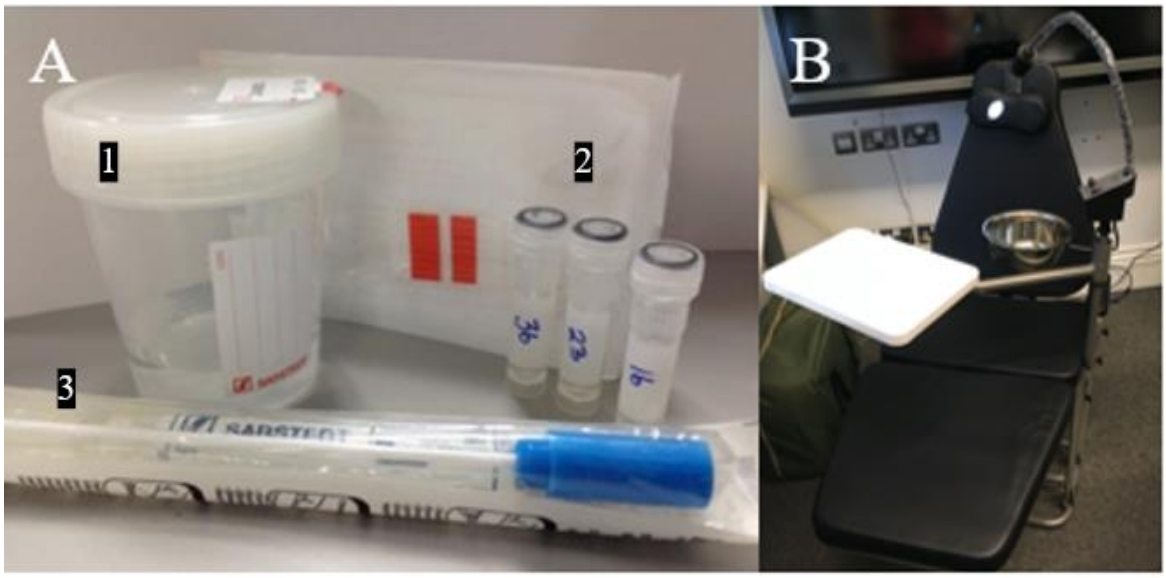


Figure 4.1. Panel A: Sample collection kits used in St. James's Hospital to obtain clinical samples from patients with Type II Diabetes with and without DFUs. This includes; 1. A 100 ml sterile collection cup (Sarstedt) containing 25 ml phosphate-buffered saline (PBS) for the oral rinse (OR) sample; 2. Periopaper™ kits and screw capped tubes containing 1 ml TSB for periodontal pocket samples (PPs), and; 3. Sterile swabs for swabs of the nares (N), a finger (F), a toe (T) and an ulcer (U), if present. Panel B: Portable dental chair used for periodontal examination and clinical sample collection onsite in the Diabetes Day Centre at St. James's Hospital.

4.2.2.2. Diabetic foot ulcer assessment and sample collection

A specialist clinical podiatrist examined the foot of each participant. Any ulcers present underwent assessment according to the Texas wound classification scheme (264). The number of ulcers present on the foot was recorded as were any prior DFU-related interventions such as surgical debridement, amputation, history of and current antibiotic usage, and if the ulcer aetiology was neuropathic, ischaemic or neuroischaemic. The age of the oldest ulcer was recorded, and this ulcer was swabbed by rotating a pre-moistened nitrogen-gassed VI-packed sterile transport swab (Sarstedt AG & Co.) in the deepest part of the ulcer for 30 s.

4.2.2.3. Relevant participant medical history

Relevant medical information recorded for each participant consisted of patient sex, age range, smoking history, number of years since diabetes diagnosis, alcohol consumption, frequency of toothbrushing, use of interdental cleaning devices, length of time since last dental appointment, HbA1c and body mass indices. This information was collected based on discussion with each participant or from medical records with the patient's permission.

Following sampling, all samples and medical history notes were pseudonymised and immediately transported to the Microbiology Laboratory at DDUH where they were processed within four hours of collection.

4.2.3. Microbiological processing of specimens

4.2.3.1. Oral rinse samples

Oral rinse samples were processed by transferring a 1 ml aliquot from the 100 ml collection cup to a sterile 1.5 ml Eppendorf Safe-lock™ microfuge tube. This aliquot was centrifuged at 20,000 × g for one min (Centrifuge 5430, Eppendorf AF, Hamburg, Germany), after which the supernatant was removed and discarded. The remaining pellet was resuspended in 1 ml of nutrient broth (NB) and vortexed at maximum speed using a Heidolph REAX vortex (Heidolph) for one min to resuspend the pellet in solution. A 100 µl aliquot of this cell suspension was used to inoculate a Sa*Select*™ chromogenic agar plates (BioRad), followed by incubation at 37°C for 24 h in a static incubator (Gallenkamp).

4.2.3.2. Periodontal pocket samples

Screw-capped collection tubes containing the PerioPaper™ strip samples suspended in TSB were vortexed at maximum speed for one min, and 100 µl aliquots of the resulting cell

suspension were plated onto Sa*Select*™ chromogenic agar plates. Inoculated plates were incubated at 37°C for 24 h in a static incubator (Gallenkamp).

4.2.3.3. Swab samples of nares, fingers, toes and ulcers

Nasal, finger, toe, and ulcer (if applicable) swabs were used to lawn the entire surface of separate Sa*Select*™ plates. Inoculated plates were incubated at 37°C for 24 h prior to examination for the presence of presumptive staphylococcal species.

4.2.3.4. Prevalence of MRSA in clinical specimens

To investigate samples for the presence of MRSA, 10 full sets of clinical samples (oral rinse, periodontal pockets samples and swabs from the nares, finger, toe and ulcer) from patients with ulcers (U67-U76) were used to inoculate MRSA*Select* II (Bio-Rad) agar plates as previously described for the inoculation of the Sa*Select*™ plates in Chapter 2, Section 2.8.1. The plates were incubated at 37°C for 24 h prior to examination for the presence of presumptive MRSA isolates based on their pink colony morphology.

4.2.4. Recovery and presumptive identification of staphylococcal species

Plates with staphylococcal growth were quantified according to colony morphology. Recovered staphylococcal isolates were presumptively identified according to colony colour and morphology on Sa*Select*™ (BioRad) following incubation at 30°C for 48 h as previously described in Chapter 2, Section 2.8.1.

4.2.5. Definitive identification of staphylococcal isolates

Presumptive *S. aureus* isolates identified based on colony colour on Sa*Select*™ agar were further tested using latex agglutination testing for identification of *S. aureus* as described in Chapter 2, Section 2.8.2. Other species, including *S. epidermidis,* were definitively identified by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and species identification by sequence analysis of the 16S rRNA gene as previously described in Chapter 2, Sections 2.8.3 and 2.8.4, respectively, were used for definitive identification of staphylococcal species. Isolates presumptively identified via species-specific colony morphologies as distinct staphylococcal species on Sa*Select*™ were selected for definitive identification. Representative colonies were carefully selected to provide an accurate representation of the various staphylococcal species recovered from each anatomical site and from each participant in the study.

4.2.6. Statistical analyses

Statistical tests including Mann-Whitney, Krushal-Wallis, median tests, Phi and Cramer tests were performed in SPSS version 25. Chi-square tests were performed using a Graphpad calculator (https://www.graphpad.com/quickcalcs/contingency1/).

4.3. Results

4.3.1. Patient cohorts recruited.

Of the patients recruited for the study (76 patients with DFUs and 76 without DFUs), 111/152 (73.0%) were male and 41/152 (27.0%) were female (Table 4.1). Of the patients without DFUs, 44/76 (58.0%) were male, and 32/76 (42.0%) were female and 67/76 (88.0%) and 9/76 (12.0%) of the patients with DFUs, were male or female, respectively. The majority of patients recruited for this study were more than 55 years of age (*N*=126, 82.8%) at the time of participation (Table 4.1).

Participants had most commonly been diagnosed with diabetes between 10 and 14 years prior to study participation (53/152 [34.2%]). Eighteen participants were diagnosed > 5 years prior to the study (18/152 [11.8%]). Twelve participants received their diagnosis between 5-10 years prior to the current investigation (12/152 [7.8%]). The second largest cohort of participants whom received their type II diabetes diagnosis was between 15-20 years before the study (34/152 [22.3%]). Finally, 22 participants were diagnosed with type II diabetes > 20 years prior to the current study (22/152 [14.4%]).

A majority of participants consisted of individuals with no (86/152 [56.5%]), or a former (49/152 [32%]) history of smoking, and predominantly reported consuming less than 5 units of alcohol per week (82/152 [53.9%]) (Table 4.1). Participants with HbA1c levels < 53 mmol/mol were classified as having ‘good’ HbA1c levels whereas participants with HbA1c levels between 54-75 mmol/mol and > 76 mmol/mol were classified as having ‘inadequate’ and ‘poor’ HbA1c levels, respectively. The mean BMI of participants with good HbA1c levels was 33 kg/m2 (range 21-48). Participants with inadequate glycaemic control had a mean BMI of 32, and participants with poor glycaemic control also had a mean BMI of 32 (Table 4.1).

Table 4.1. Demographic variables of participants with type II diabetes (*N*=152) included in the present investigation

Patient Sub-category		*N* (%)			
HbA1c Category (mmol/mol)		**Good (< 53)**	**Inadequate (54 – 75)**	**Poor (> 76)**	**Unknown[b]**
Gender	Male	29 (26.4)	36 (32.7)	18 (16.4)	28 (25.2)
	Female	14 (34.1)	13 (31.7)	8 (19.5)	6 (14.6)
Age range	35-44	2 (16.7)	6 (50)	1 (8.3)	3 (25)
	45-54	6 (42.9)	3 (21.4)	1 (7.1)	4 (28.6)
	55-64	12 (24.5)	13 (26.5)	12 (24.5)	13 (26.0)
	65-74	16 (33.3)	17 (35.4)	8 (16.7)	7 (14.6)
	>74	7 (26.9)	8 (30.8)	4 (15.4)	7 (26.9)
	Unknown	0 (0)	2 (100)	0 (0)	0 (0)
Years since diabetes diagnosis	< 5	7 (38.9)	6 (33.3)	2 (11.1)	3 (16.7)
	5-10	6 (50)	1 (8.3)	4 (33.3)	1 (8.3)
	10-14	17 (32.7)	15 (28.8)	6 (11.5)	15 (27.0)
	15-20	7 (20.6)	13 (38.2)	8 (23.5)	6 (17.6)
	> 20	3 (13.6)	11 (50)	1 (4.5)	7 (31.8)
	Unknown	3 (23.1)	3 (23.1)	5 (38.5)	2 (15.4)
Smoking status	Never	28 (32.6)	27 (31.4)	14 (16.3)	17 (19.8)
	Former	15 (31.3)	18 (37.5)	7 (14.6)	9 (18.4)
	Current	0 (0)	4 (25)	4 (25)	8 (50)
	Unknown	0 (0)	0 (0)	1 (100)	0 (0)
Alcohol consumption	Low (<5 units/week)	25 (30.9)	28 (34.6)	23 (28.4)	6 (7.3)
	Moderate (5-14 units/week)	7 (46.7)	6 (40)	1 (6.7)	1 (6.7)
	High (<14 units/week)	1 (10)	5 (50)	1 (10)	3 (30)
	Unknown	10 (22.2)	10 (22.2)	1 (2.2)	24 (53.3)
Ulcer status	No ulcer	25 (33.3)	23 (30.7)	18 (24)	10 (13.2)
	Ulcer	18 (23.7)	26 (34.2)	8 (10.5)	24 (31.6)
Comparison of average numbers of natural teeth, BMI and HbA1c between each patient cohort					
Mean number of natural teeth (range)		13 (0-31)	17 (0-31)	15 (0-28)	15 (4-23)
Mean BMI (kg/m2) (range)		33 (21-48)	32 (17-49)	32 (23-44)	32 (30-35)
Mean HbA1c (mmol/mol) (range)		45 (35-53)	63 (54-74)	95 (76-148)	NA

[a] Colony counts were determined as colony forming units (CFU)/ml for oral rinse and periodontal pocket samples, and as CFU/swab for ulcer, finger, toe and nasal swabs.

[b] HbA1c measurements were not available for these patients.

Abbreviations: BMI; Body Mass Index, HbA1c; glycated haemoglobin concentration, NA; Not applicable.

4.3.2. Oral and periodontal health of participants

A complete periodontal examination was performed on the 76 patients without DFUs, however, due to participants feeling uncomfortable with an oral exam or unavailability of a dental professional to perform a periodontal assessment, only 54/76 patients with DFUs underwent periodontal examination. The 22 patients with DFUs who did not undergo periodontal examination provided self-reported details regarding the number of natural teeth they had, any dental prostheses they used and the frequency of their oral hygiene routines. Two of the patients with DFUs had no natural teeth and therefore periodontal examination could not be performed. Bleeding on probing was observed in 54/76 (71.1%) of patients without DFUs, and 37/54 (68.5%) patients with DFUs who underwent periodontal examination (Table 4.2).

The majority of participants reported that they brushed their teeth at least once per day (*N*=125 [82.2%]), however a noticeable difference in the regularity of toothbrushing was apparent between patient groups with and without DFUs. Of the patients with DFUs, 71% (*N*=54) reported brushing their teeth once a day or less. In contrast, of the patients without DFUs, only 46% (*N*=35) reported brushing their teeth once per day or less with the majority of this patient group (*N*=41, 53.9%) brushing twice per day, or more frequently (Table 4.2).

Regularity of visits to dental professionals was sub-optimal for both participant groups. Participants with (51/76 [67.1%]) and without (51/76 [67.1%]) DFUs reported they had not visited their dentist > 12 months prior to sampling (Table 4.2). No obvious differences between mean number of natural teeth or periodontal pocket depth were observed between the two participant groups (Table 4.2). In contrast, the mean plaque score was higher in participants with DFUs (mean: 72.0%) than in participants without DFUs (mean: 61.0%)**,** however this difference was not significant (*P*>0.99363) (Table 4.2).

Table 4.2. Oral health and hygiene routine of patients without ulcers (*N*=76) and patients with ulcers (*N*=76)

Patient group and oral health/hygiene subcategory[a]		Ulcer absent *N (%)*	Ulcer present *N (%)*
Gender*			
	Male	44 (57.9)	67 (88.2)
	Female	32 (42.1)	9 (11.8)
Bleeding on probing*			
	No	19 (25.0)	16 (21.1)
	Yes	50 (65.8)	37 (48.7)
	No teeth	5 (6.6)	1 (1.3)
	Unknown	2 (2.6)	22 (28.9)
Suppuration*			
	None	60 (78.9)	42 (77.8)
	Yes	3 (3.9)	0 (0)
	NA (No teeth present)	5 (6.6)	3 (5.5)
	Unknown	8 (10.5)	31 (57.4)
Regularity of brushing*			
	< Once per day	11 (14.5)	16 (21.1)
	Once per day	24 (31.6)	38 (50)
	≥ Twice per day	41 (53.9)	22 (28.9)
Interdental care			
	No	64 (84.2)	63 (82.9)
	Yes	12 (15.8)	13 (17.1)
Last dental appointment			
	< 3 mths previously	14 (18.4)	9 (11.8)
	3-6 mths previously	3 (3.9)	3 (3.9)
	6-12 mths previously	8 (10.5)	13 (17.1)
	> 12 mths previously	51 (67.1)	51 (67.1)
Presence/type of prostheses			
	None	42 (55.3)	53 (69.7)
	Dentures	31 (40.8)	19 (25)
	Bridge	2 (2.6)	1 (1.3)
	Crowns	0 (0.0)	2 (2.6)

Implants	1 (1.3)	1 (1.3)

Mean (Range) of diabetes control and periodontal health of participants

	Ulcer absent Mean (range)	**Ulcer present Mean (Range)**
HbA1c (mmol/mol)	65 (36-148)	62 (35-112)
Body Mass Index	33 (24-49)	32 (17-45)
Number of natural teeth	15 (0-29)	15 (0-31)
Clinical attachment loss	8 (4-15)	8 (3-11)
[c]Periodontal pocket site A **	6 (3-10)	5 (2-8)
Periodontal pocket site B**	5 (3-8)	5 (2-8)
Plaque Score	61 (0-100)	72 (0-100)

[a] Statistical significance (P<0.05) according to Chi-square comparison of categorical variable distribution is indicated by the presence of a single asterisk in the relevant patient sub-category title. Statistical significance (P<0.05) determined by Mann-Whitney tests for independent samples are indicated by the presence of two asterisks in the relevant patient sub-category title.

[b] Periodontal probing was not conducted for 22 patients with ulcers who participated in the study.

[C] Periodontal pocket site A was sampled with PerioPaper™ for 60 s, and site B was sampled for 30 s.

Abbreviations: NA; Not applicable, HbA1c; glycated haemoglobin concentration, mths; months.

4.3.3. Foot health and ulcer status of participants

Half of the 152 study participants had active DFUs at the time of participation (N=76). A Chi-square test revealed a significantly higher proportion of males in the ulcer group (P<0.05) (Table 4.3). The predominant ulcer type was neuropathic (59/76 [77.6%]) and the remainder were either neuroischaemic (14/76 [18.4%]) or ischaemic (3/76 [3.9%]). The predominant ulcer grades were identified as type 1A (superficial wound, pre-ulcerative and intact skin (19)) (53/76 [69.7%]). Based on the Texas Wound Classification criteria, clinical signs of infection were observed in 9/76 (11.8%) of the DFUs sampled. The average number of active ulcers was one per patient (range 1-3). The average DFU age was 25 weeks (range 1-116 weeks). Prior LEA history was recorded for 21/76 (27.6%) of the participants with DFUs (Table 4.3).

Table 4.3. Clinical features of ulcers from which samples (*N*=76) were collected

Clinical ulcer presentation	Ulcer sub-category	*N*	%
Ulcer type	Neuropathic	59	77.6
	Neuroischaemic	14	18.4
	Ischaemic	3	3.9
Texas classification[a]	1A	53	69.7
	1B	3	3.9
	1D	2	2.6
	2A	1	1.3
	3A	0	0.0
	3B	2	2.6
	3D	1	1.3
	1C	7	9.2
	2B	1	1.3
	2C	1	1.3
	0A	3	3.9
Prior amputation history	None	42	55.3
	Prior amputation	21	27.6
	Unknown	13	17.1
Sex	Male	67	88.2
	Female	9	11.8

Patient history	Mean	SEM	Range
No. active ulcers	1	0	(1-3)
Age of oldest ulcer (weeks)	25	4	(1-119)
BMI	32	1	(17-45)
HbA1c	62	3	(35-112)

[a]Texas classification refers to the depth of the ulcer: grade 1 (superficial wound not involving tendon, capsule, or bone), grade 2 (wound penetrating to tendon or capsule), and grade 3 (wound penetrating bone or joint); A (pre-ulcerative lesions with minimal to no skin break), B (with infection), C (with ischaemia), and D (with infection and ischaemia).

4.3.4. Abundance of staphylococcal species in anatomical sites of participants sampled

Comparative staphylococcal bioburdens were determined from samples collected from all anatomical sites investigated in patients both with and without DFUs (*N*=152) (Table 4.4). Staphylococci were recovered in higher abundance from patients with DFUs than patients without DFUs from all anatomical sites (oral cavity, nares, periodontal pockets, finger, toe). Patients without DFUs harboured a higher average density of staphylococci in periodontal pockets (mean = 48, range 0-3000 CFU/ml) than patients with DFUs (mean = 45, range 0-2032 CFU/ml). The density of staphylococci recovered was nearly three-fold higher on the skin of the finger (mean = 65, range 0-1244 CFU/swab) and toe (mean = 731, range 0-8060 CFU/swab) from patients with DFUs than from the finger (mean = 23, range 0-403 CFU/swab) and toe (mean =260, range 0-3000 CFU/swab) of patients without DFUs, respectively. However, equal variance *t*-tests comparing the means between each patient group for each distinct anatomical site detected no significant differences in CFU/sample between patients with and without DFUs. In both patient groups, staphylococci were most abundant in the anterior nares (NU patients: mean = 547, range 0-2772 CFU/swab, U patients: mean 724, range 0-8505 CFU/swab). (Table 4.4). Of all the patients and sites samples, the highest mean abundance was detected in the ulcer sites of patients with DFUs (mean = 831, range 0-9890 CFU/swab) (Table 4.4).

Table 4.4. Comparative staphylococcal colony counts recovered from samples collected from different anatomical sites in patients with type II diabetes with, and without ulcers.

Sample type (CFU/sample)	CFU Range	*N* (%)	
		Ulcer absent (*N* = 76)	Ulcer present (*N* = 76) [a]
Oral rinse CFU/ml	0	13 (17.1)	9 (11.8)
	1 - 49	31 (40.8)	31 (40.8)
	50 - 99	11 (14.5)	9 (11.8)
	100 - 499	19 (25)	24 (31.6)
	>500	2 (2.6)	3 (3.9)
	Mean[b]	99	119
	Range	0 -1243	0-1432
Nares CFU/swab	0	4 (5.3)	12 (15.8)
	1 - 49	14 (18.4)	9 (11.8)
	50 - 99	6 (7.9)	7 (9.2)
	100 - 499	23 (30.3)	24 (31.6)
	>500	29 (38.2)	24 (31.6)
	Mean [b]	547	724
	Range	0-2772	0-8505
Periodontal pocket A[a] CFU/ml	0	37 (48.7)	20 (37.3)
	1 - 49	37 (48.7)	32 (59.3)
	50 - 99	1 (1.3)	1 (1.8)
	100 - 499	1 (1.3)	0 (0)
	>500	0 (0)	1 (1.8)
	Mean [b]	6	24
	Range	0-122	0-872
Periodontal pocket B [a] CFU/ml [c]	0	37 (48.7)	20 (37.0)
	1 - 49	35 (46.1)	29 (53.7)
	50 - 99	2 (2.6)	3 (5.6)
	100 - 499	1 (1.3)	1 (1.85)
	>500	1 (1.3)	1 (1.85)
	Mean [b]	48	45
	Range	0-3000	0 - 2032

Finger CFU/swab [d]	0	24 (31.6)	17 (22.4)
	1 - 49	42 (55.3)	47 (61.8)
	50 - 99	6 (7.9)	3 (3.9)
	100 - 499	4 (5.3)	6 (7.9)
	>500	0 (0)	3 (3.9)
	Mean[b]	23	65
	Range	0 - 403	0 - 1244
Toe CFU/swab	0	8 (10.5)	17 (22.4)
	1 - 49	33 (43.4)	22 (28.9)
	50 - 99	6 (7.9)	8 (10.5)
	100 - 499	18 (23.7)	13 (17.1)
	>500	11 (14.5)	16 (21.1)
	Mean[b]	260	731
	Range	0 - 3000	0 - 8060
Ulcer CFU/swab	0	NA	4 (5.2)
	1 - 49	NA	15 (19.7)
	50 - 99	NA	0 (0)
	100 - 499	NA	8 (10.5)
	>500	NA	49 (64.4)
	Mean[b]	NA	831
	Range	NA	0 - 9890

Abbreviations: CFU; Colony forming units, NA; Not applicable.

[a] Periodontal pockets were not sampled from 22 patients with ulcers who participated in the study. These mean CFU/ml calculations were based on the periodontal pockets of 54 participants with ulcers sampled.

[b] *P* values are based on independent *t*-tests assuming equal variance, comparing the means between each patient group for each distinct anatomical site. No significant differences in CFU/sample were detected between patients with and without ulcers.

[c] CFU/ml indicates the number of colony-forming units obtained in 1 ml of liquid (TSBor sterile saline) used for sample collection.

[d] CFU/swab indicates the number of colony-forming units obtained in one swab sample (nares, finger, toe or ulcer) when lawned across the surface of a Sa*Select*™ agar plate.

4.3.5. Prevalence of staphylococcal species in anatomical sites of participants sampled.

4.3.5.1. Summary of species subjected to definitive identification.

A total of 3176 presumptive staphylococcal isolates recovered from patients with and without DFUs were stored as described in Chapter 2, Section 2.1. Where present, a minimum of three red-coloured colonies recovered on Sa*Select*™ agar (indicative of *S. aureus*) were stored as presumptive *S. aureus* and at least four pink/white colonies recovered on the same medium (indicative of *S. epidermidis*) were stored as presumptive *S. epidermidis* isolates in order to recover multiple distinct isolates of each species, if present. A total of 1677 isolates were stored from multiple distinct anatomical sites of patients with DFUs (*N*= OR:343, N:380, PP:187, F:175, T:233, U:357]), and 1501 isolates were stored from multiple distinct anatomical sites of patients without DFUs (*N*= OR:387, N:441, PP: 220, F:178, T:275]). A total of 2397 isolates were selected for definitive species identification based on species-specific colony colour on Sa*Select*™ as described in Chapter 2, Section 2.8. Multiple isolates with similar colony morphologies recovered from multiple distinct sites for each participant were selected for identification, ensure accurate representation of distinct species from each anatomical site per participant investigated. Of these isolates identified, 913 were from patients without DFUs (OR:228/913 [24.9%], N:369/913 [40.4%], PP:113/913 [12.3%], F:95/913 [10.4%], T:108/913 [12.0%]) and 1484 were from patients with DFUs, (OR:324/1484 [21.8%], N:326/1484 [21.9%], PP:177/1484 [11.9%%], F:162/1484 [11.1%%], T:199/1484 [13.5%], U:296/1484 [19.9%]).

Of the patients without DFUs, the isolates recovered from the oral cavity were predominantly *S. aureus* (108/228, 47.3%), *S. epidermidis* (92/228 [40.3%]), and other CoNS species (28/228 [12.2%]) (Table 4.5). Anterior nares isolates were identified as *S. aureus* (116/369 [31.4%]), *S. epidermidis* (124/369 [33.6%]), and other CoNS species (129/369 [34.9%]). Isolates from the periodontal pockets were identified as *S. aureus* (34/113, 30.1%), *S. epidermidis* (56/113 [49.5%]) and other CoNS species (23/113 [20.3%]). Isolates from the finger were identified as *S. aureus* (14/95 [14.8%]), *S. epidermidis* (46/95 [48.4%]) and other CoNS species (35/95 [36.8%]), and from the toe as *S. aureus* (4/108 [3.7%]), *S. epidermidis* (25/108 [23.1%]) and other CoNS species (79/108 [73.2%]) (Table 4.5).

Table 4.5. Species identification of staphylococcal species from multiple anatomical sites from patients with and without DFUs.

Anatomical Site	*S. aureus* (%)	*S. epidermidis* (%)	Other CoNS[a] (%)
Participants without DFUs			
Oral cavity	108/228 (47.3%)	92/228 (40.3%)	28/228 (12.2%)
Nares	116/369 (31.4%)	124/369 (33.6%)	129/369 (34.9%)
Periodontal pockets	34/113 (30.1%)	56/113 (49.5%)	23/113 (20.3%)
Finger	14/95 (14.8%)	46/95 (48.4%)	35/95 (36.8%)
Toe	4/108 (3.7%)	25/108 (23.1%)	79/108 (73.2%)
Participants with DFUs			
Oral cavity	139/324 (42.9%)	145/324 (44.7%)	40/324 (12.4%)
Nares	147/326 (45.1%)	128/326 (39.2%)	51/326 (15.6%)
Periodontal pockets	34/177 (19.2%)	95/177 (53.6%)	48/177 (27.1%)
Finger	27/162 (16.5%)	53/162 (32.5%)	83/162 (50.9%)
Toe	23/199 (13.3%)	76/199 (32.6%)	126/199 (54.1%)
Ulcer	176/357 (49.2%)	31/296 (8.6%)	89/296 (42.2%)

[a]For the purpose of this investigation this does not include isolates of *S. epidermidis.*

The prevalence of *S. aureus* was also higher on the finger of patients with DFUs (10/76 [13.2%]) than those without DFUs (4/76 [5.3%]) and was significantly higher ($P<0.05$) on the toe skin patients with DFUs (10/76 [13.2%]) than patients without DFUs (3/76 [3.9%]). *Staphylococcus aureus* was recovered from the DFUs of 39/76 patients (51.3%) (Table 4.2). Methicillin-resistant *S. aureus* was not detected from any of the 10 sets of clinical samples collected from patients with DFUs (U67-U76) using MRSA*Select*™ medium.

Of the patients with DFUs, isolates recovered from the oral cavity were predominantly *S. aureus* (139/324 [42.9%]), *S. epidermidis* (145/324 [44.7%]), and other CoNS (40/324 [12.4%]). The nasal cavity isolates were identified as *S. aureus* (147/326 [45.1%]), *S. epidermidis* (128/326 [39.2%]), and other CoNS species (51/326 [15.6%]). Isolates from the periodontal pockets were identified as *S. aureus* (34/177 [19.2%]), *S. epidermidis* (95/177 [53.6%]) and other CoNS species (48/177 [27.1%]). Isolates from the

finger were identified as *S. aureus* (27/162 [16.5%]), *S. epidermidis* (53/162 [32.5%]) and other CoNS species (83/162 [50.9%]), and from the toe as *S. aureus* (23/199 [13.3%]), *S. epidermidis* (76/199 [32.6%]) and other CoNS species (126/199 [54.1%]). Isolates from the DFUs of this cohort of patients were identified as *S. aureus* (176/357 [49.2%]), *S. epidermidis* (31/296 [8.6%]) and other CoNS species (89/296 [42.2%]) (Table 4.5).

The other, non-*S. epidermidis* CoNS species recovered from both patient groups were identified as: *S. capitis* (93/2397 [3.8%]); *S. hominis* (35/2397 [1.5%]); *S. saprophyticus* (52/2397 [2.2%]); *S. haemolyticus* (94/2397 [3.9%]); *S. caprae* (32/2397 [1.4%]) *S. lugdunensis* (18/2397 [0.7%]), *S. pasteuri* (21/2397 [0.8%]); *S. pettenkoferi*, (16/2397 [0.6%]); *S. kloosi* (1/2397 [0.03%]), *S. pseudointermidus* (1/2397 [0.03%]); *S. sciuri* (3/2397 [0.1%]), *S. warneri* (14/2397 [0.5%]); *S. arlettae* (4/2397 [0.1%]), *S. ureilyticus* (34/2397 [1.4%]), *S. cohnii* (31/2397 [1.3%]); *S. carnosus* (2/2397 [0.1%]).

4.3.5.2. Prevalence of S. aureus *amongst patients with and without DFUs*

Staphylococcus aureus was recovered from at least one anatomical site of 34/76 (44.7%) patients without DFUs (Table 4.6), and from the oro-nasal cavities of 31/76 (40.7%) patients without DFUs (OC:26/76 [34.2%], N:12/76, [15.7%], PP:9/76 [6.5%]). *Staphylococcus aureus* was significantly more prevalent in patients with DFUs ($P<0.03$) and was recovered from at least one anatomical site of patients with DFUs in 62/76 participants (81.5%). The corresponding prevalence of *S. aureus* in patients without DFUs was significantly lower, with *S. aureus* recovered from at least one anatomical site in 36/76 (47.3%) participants. This species was recovered from the oro-nasal sites of 60/76 (78.9%) patients with DFUs (OC:35/76 [46.0%], N:36/76 [47.3%], PP:13/54 [24.1%]). Oro-nasal carriage of *S. aureus* was also significantly higher in patients with DFUs (60/76 [78.9%]) than patients without DFUs (31/76 [40.7%]) ($P<0.05$).

Table 4.6. Prevalence of staphylococcal species in distinct anatomical sites of participants with diabetes, with (*N*=76) [a] and without (*N*=76) diabetic foot ulcers.

Staphylococcal species	Nasal swab	Oral Cavity	PP [a]	Finger	Toe	Ulcer
			Participants with DFUs (*N* (%))			
S. aureus	12 (15.8)	9 (11.8)	2 (3.7)	2 (2.6)	2 (2.6)	21 (27.6)
S. aureus and CoNS[b]	3 (3.9)	2 (2.6)	1 (1.9)	2 (2.6)	3 (3.9)	13 (17.1)
S. aureus and mixed CoNS	1 (1.3)	1 (1.3)	0 (0)	2 (2.6)	3 (3.9)	2 (3.9)
S. aureus and non-staphylococcal species	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.3)
S. aureus, CoNS and non-staphylococcal species	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.3)	1 (1.3)
S. aureus and *S. epidermidis*	13 (17.1)	15 (19.7)	8 (14.8)	3 (3.9)	2 (2.6)	0 (0)
S. aureus, *S. epidermidis* and other CoNS	8 (10.5)	8 (10.5)	2 (3.7)	1 (1.3)	0 (0)	0 (0)
S. epidermidis	15 (19.7)	18 (23.7)	15 (27.8)	7 (9.2)	7 (9.2)	1 (1.3)
S. epidermidis and other CoNS	7 (9.2)	6 (7.9)	7 (12.9)	15 (19.7)	8 (10.5)	5 (6.6)
S. epidermidis and mixed other CoNS	1 (1.3)	2 (2.6)	2 (3.7)	3 (3.9)	2 (2.6)	2 (2.6)
S. epidermidis and non-staphylococcal species	1 (1.3)	1 (1.3)	0 (0)	1 (1.3)	0 (0)	2 (2.6)
CoNS and non-staphylococcal species	1 (1.3)	2 (2.6)	0 (0)	1 (1.3)	0 (0)	3 (3.9)
Mixed CoNS species	2 (2.6)	0 (0)	0 (0)	5 (6.6)	15 (19.7)	5 (6.6)
One CoNS species	11 (14.5)	9 (11.8)	9 (16.7)	22 (28.9)	22 (28.9)	11 (14.5)
Non-staphylococcal species	0 (0)	0 (0)	1 (1.9)	3 (3.9)	2 (2.6)	3 (3.9)
Negative culture	1 (1.3)	3 (3.9)	7 (16.1)	9 (11.8)	9 (11.8)	3 (3.9)
Total *S. aureus* positive	36 (47.4)	35 (46.1)	13 (24.1)	10 (13.2)	11 (14.5) [b]	38 (51.3)
Total *S. epidermidis* positive	45 (59.2)	56 (73.6) [b]	34 (62.9) [b]	30 (39.5) [b]	19 (25)	10 (13.2)

	Participants without DFUs (*N* (%))					
S. aureus	12 (15.8)	8 (11)	4 (5.3)	0 (0)	1 (1.3)	NA
S. aureus and CoNS[b]	5 (6.6)	3 (4)	1 (1.3)	3 (3.9)	1 (1.3)	NA
S. aureus and mixed CoNS	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
S. aureus and non-staphylococcal species	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	NA
S. aureus, CoNS and non-staphylococcal species	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
S. aureus and *S. epidermidis*	4 (5.3)	8 (11)	2 (2.6)	0 (0)	1 (1.3)	NA
S. aureus, *S. epidermidis* and CoNS	5 (6.6)	5 (7)	2 (2.6)	1 (1.3)	0 (0)	NA
S. epidermidis	20 (26.3)	13 (17)	16 (21.1)	10 (13.2)	6 (7.9)	NA
S. epidermidis and CoNS	10 (13.2)	10 (13)	6 (7.9)	6 (7.9)	7 (9.2)	NA
S. epidermidis and mixed CoNS	0 (0)	0 (0)	0 (0)	0 (0)	2 (2.6)	NA
S. epidermidis and non-staphylococcal species	0 (0)	0 (0)	1 (1.3)	0 (0)	0 (0)	NA
CoNS and Non-staphylococcal species	0 (0)	1 (1)	1 (1.3)	0 (0)	1 (1.3)	NA
Mixed CoNS	1 (1.3)	0 (0)	1 (1.3)	2 (2.6)	13 (17.1)	NA
One CoNS	16 (21.1)	22 (29)	25 (32.9)	40 (52.6)	39 (51.3)	NA
Non-staphylococcal species	0 (0)	1 (1)	0 (0)	0 (0)	1 (1.3)	NA
Negative culture	3 (3.9)	4 (5)	17 (22.4)	14 (18.4)	4 (5.3)	NA
NA	0	0	0	0	0	76 (100)
Total *S. aureus* positive	26 (34.2)	25 (32.9)	9 (11.8)	4 (5.3)	3 (3.9) [c]	0 (0)
Total *S. epidermidis* positive	39 (51.3)	36 (47.4)	27 (35.5) [c]	17 (22.4) [c]	16 (21.1)	0 (0)

[a] Periodontal pocket datasets are based on 54 patients with DFUs as 22 patients with DFUs did not have periodontal pockets sampled. All 76 participants with DFUs had samples taken from all the other anatomical sites investigated.

[b] CoNS does not include *S. epidermidis* for the purpose of the present investigation.

[c] Two-tailed Chi-square tests revealed that the prevalence of *S. aureus* was significantly higher on the toe-skin of partcipants with DFUs, and the prevalence of *S. epidermidis* was significantly higher in the periodontal pockets and on the finger skin of participants with DFUs.

Abbreviations: PP; Periodontal pockets, DFUs; Diabetic foot ulcers, NA; Not applicable.

4.3.5.3. Prevalence of S. epidermidis *in patients with and without DFUs*

Staphylococcus epidermidis was recovered from at least one anatomical site of 65/76 (86%) patients without DFUs (Table 4.5), and from the oro-nasal sites of 62/76 (81.5%) patients without DFUs (OC:36/76 [47.4%], N:39/76 [51.3%], PP:27/76 [35.5%]). In contrast, *S. epidermidis* was recovered from at least one anatomical site of 70/76 (92.1%) patients with DFUs. From this patient cohort, *S. epidermidis* was recovered from the oro-nasal sites of 70/76 (92.1%) patients (OC:56/76 [73.6%], N:53/76 [69.7%], PP:34/54 [62.9%]). There was no significant difference between the oro-nasal prevalence of *S. epidermidis* between the two patient cohorts, however, the prevalence of *S. epidermidis* was significantly higher on the fingers of patients with DFUs (35/76 [46.1%]) than patients without DFUs (17/76 [22.4%]) (P<0.03). The toe skin of 18/76 (23.6%) and 20/76 (26.3%) patients with and without DFUs was colonised by *S. epidermidis*, respectively, however no significant difference was identified. The ulcers of 10/76 (13.2%) patients with DFUs yielded *S. epidermidis* (Table 4.6).

4.3.5.4. Prevalence of other CoNS in patients with and without DFUs

Coagulase-negative staphylococci were recovered from at least one anatomical site of all 76/76 (100%) patients investigated, both with and without DFUs. Isolates were recovered from the oro-nasal sites of 53/76 (69.7%) patients without DFUs (OC:30/76 [39.4%], N:26/76 [34.2%], PP:27/76 [35.5%]) and 63/76 (82.8%) patients with DFUs (OC:59/76 [77.6%], N:61/76 [80.2%], PP:36/54 [66.7%]). The prevalence of CoNS was significantly higher in the nares of patients with DFUs (61/76 [80.2%]) than patients without DFUs (26/76 [34.2%]) (P<0.05).

The predominant CoNS species other than *S. epidermidis* identified from the ONC of non-DFU patients were *S. haemolyticus* (OC: [N=6], N: [N=6], and *S. capitis* (PP: [N=5]). The same two species were the predominant CoNS species other than *S. epidermidis* species recovered from the ONC of patients with DFUs; *S. haemolyticus* (N: [N=14]) and *S. capitis* (OC: [N=5], PP: [N=6]).

The fingers of 45/76 (59.2%) and 55/76 (72.3%) of patients with and without DFUs were colonised with CoNS species other than *S. epidermidis*, respectively. The most predominant species recovered were *S. hominis* (N=6) and *S. capitis* (N=11), respectively. There was no significant difference observed between CoNS species other than *S. epidermidis* between the two patient cohorts.

The toe of 59/76 (77.6%) and 62/76 (81.5%) of patients with and patients without DFUs were colonised with CoNS species other than *S. epidermidis*, respectively. The

predominant species recovered from patients without DFUs was *S. saprophyticus* (*N*=22), whereas the predominant species among the patients with DFUs were *S. saprophyticus* (*N*=6) and *S. caprae* (*N*=6). There were no significant differences detected between CoNS species other than *S. epidermidis* prevalence on the toe between the two patient groups. Coagulase-negative staphylococci other than *S. epidermidis* were also recovered from the DFUs of 50/76 (65.7%) patients in this group. The most commonly identified species other than *S. epidermidis* recovered from DFUs was *S. haemolyticus* (*N*=10).

4.3.6. Identification of the same staphylococcal species in the oro-nasal cavity and ulcer sites of patients with DFUs

The same staphylococcal species, including *S. aureus*, *S. epidermidis* and other distinct CoNS were identified in both the oro-nasal and ulcer sites of 38/76 (50.0%) patients with DFUs (Table 4.7). The predominant species identified in both oro-nasal and ulcer sites was *S. aureus*, which was recovered from both sites of 26/76 (34.2%) patients with DFUs (U3, U7, U12, U14, U15, U18, U21, U25, U26, U33, U34, U40, U41, U43, U44, U47, U48, U51, U55-U58, U61, U63, U67 and U74). From this cohort of patients, *S. aureus* was recovered from the nares and DFUs of 22/26 (78.5%) patients, the oral cavity and DFUs of 22/26 (78.5%), and the periodontal pockets and DFUs of 6/26 (21.4%) patients. *Staphylococcus aureus* was recovered from multiple oro-nasal sites of 17/26 (60.7%) patients in this group (U3 [OC, N], U12 [OC, N, PP], U18 [OC, PP], U21 [OC, N, PP], U25 [OC, N], U26 [OC, N], U34 [OC, N], U40 [OC, N], U41 [OC, N], U44 [OC, N], U47 [OC, N], U55 [OC, N], U56 [OC, N], U57 [OC, N], U63 [OC, N], U67 [OC, N]) (Table 4.7).

Staphylococcus epidermidis, the second most frequently identified species, was recovered from both the oro-nasal and ulcer sites of 10/76 (13.2%) patients with DFUs (U1, U5, U11, U16, U24, U42, U45, U46, U49 and U50). From this patient group, *S. epidermidis* was recovered from the nares and DFU sites of 7/10 (70.0%) patients, the oral cavity and DFUs of 8/10 (80.0%) of patients and the periodontal pockets and DFUs of 9/10 (90%) patients. *Staphylococcus epidermidis* was recovered from multiple oro-nasal sites of all 10/10 (100%) patients (U1 [OC, N, PP], U5 [OC, N, PP], U11 [OC, PP], U16 [OC, N, PP], U24 [OC, N, PP], U42 [OC, N, PP], U45 [OC, N], U46 [OC, PP], U49 [OC, PP], U50 [N, PP]) (Table 4.7).

Table 4.7. Staphylococcal species identified from anatomical sites of 38 patients with diabetic foot ulcers from whom the same species was recovered from both oro-nasal and ulcer sites

Patient	Species detected in both oro-nasal and ulcer sites	Oro-nasal sites			Finger	Toe	Ulcer
		Nasal Swab	Oral Cavity	Periodontal pocket [a]			
U1	SE, SP	SE, SHo, SP	SE, SP	SE	SC, SHo	SE, SHo	SE, SP
U3	SA	SA, SE, SH	SA	SE	SE, SHo, SH, SS	SE, SS, SH, SU	SA
U5	SE	SE	SA, SE	SE, SC	SE	SE, SU, SS	SE, SU, SL
U7	SA	SE	SA	SC, SE	SC	SC, SWi	SA
U11	SE	SA	SA, SE, SU	SE	NC	SE, SH	SE, SSi
U12	SA	SA	SA	SA, SE	SA, CoNS	SE	SA, SSi
U14	SA	SA	SE	SE	SHo, SC	SA	SA
U15	SA	SE	SA, SE, SPa	SE, SPa, SC	SA	SE, SU	SA, CoNS
U16	SE	SA, SE	SA, SE	SA, SE	SE	SU	SE, SU, SH, SSu
U17	SH	SH, SC, SS	SA	SA, SE, SP	NA	SU	SH
U18	SA	SE	SE, SA	SA, SE	SE	SE, SC	SCa, SH, SA, SU
U21	SA	SA	SA	SE, SA, SS	SS, SU	SA, SE	SA
U24	SE	SE	SE	SE	CoNS	CoNS	SE
U25	SA	SA, SL	SA, SE	SC, SE, SU	SE	SCa	SA
U26	SA	SA, SE	SA, SE	SE	SE	SK, SC	SA
U33	SA	CoNS	NC	SA	CoNS	CoNS	SA
U34	SA	SA, SE, SH	SA, SE, SPa	CoNS	SE	SE	SA, SLe
U40	SA	SA	SA, CoNS	SE	SA	NC	SA, SSi
U41	SA, SS	SE, SA, SS	SE, SA	NC	SA, SC	SA, SC, SS	SA, SS, SCa
U42	SE	SE, SW, SC	SE, SC	SE, SC	SE, SC	SC	SE, SP
U43	SA	SE, SA	SE	SE, SEq, SS	SE, SA, SHo	NC	SA
U44	SA	SA	SE, SA	SE	SE, SC	SA, SP	SA
U45	SE	SE	SE, SPa	NC	SE	NC	SE, SP

U46	SE	SA	SE	SE	NC	SA, SC	SE, SC
U47	SA	SE, SA, SH	SA, SE	SA, SE	SE, SC	SE, SA	SA, SSi
U48	SA	SA	SE	SE	NC	SA	SA
U49	SE	SA, SH	SE, SH, SC	SE, SC	SE	NC	SE, SCa
U50	SE	SE	NC	SE	SHo	SE, SCa	SE, SCa
U51	SA	SE, SA, sHo	SE	NC	SE, SHo	SE	SA
U55	SA	SA, SE	SA, SE, SL	SE, SA	SH	SH	SA
U56	SA	SA, SE	SA, SE	SHo	SA, SE	SE	SA
U57	SA	SA, SS, SW	SA, SE	NA	SH	NC	SA
U58	SA	SA, SE	SEq, SW, SSu, SE	NA	SC	SU, SP	SA
U61	SA	SA	SE	NA	SA SW, SH, SU	SCa, SU	SH, SU, SA
U63	SA	SA, SE	SA, SE	NA	SA, SE	CoNS	SA, SSu
U67	SA	SA, SU	SA, SE	NA	SU	SA, SU, SAr	SA, CoNS
U72	SH	SH	SH	NA	NC	SCa	SH
U74	SA	SE	SA	NA	NC	SCr	SA

[a] These datasets are based on 76 patients without DFUs and 56 patients with DFUs as periodontal pockets were not sampled for 20/76 patients with DFUs who participated in the study.

Abbreviations: CoNS; Unidentified coagulase negative *Staphylococcus* species, NA; Not applicable, NC; Negative culture, SA; *S. aureus* (indicated in red typeface), SAr; *Staphylococcus arlettae*, SC; *Staphylococcus capitis*, SCa; *Staphylococcus caprae*, SCr; *Staphylcoccus carnosus*, SE; *S. epidermidis* (indicated in blue typeface), SEq; *Staphylococcus equorum*, SH; *Staphylococcus haemolyticus*, SHo; *Staphylococcus hominis*, SK; *Staphylococcus kloosi*, SL; *Staphylococcus lugdunesis*, SLe; *Staphylcoccus lentus*, SP; *Staphylococcus pettenkoferi*, SPa; *Staphylococcus pasteuri*, SS; *Staphylococcus saprophyticus*, SSi; *Staphylococcus simulans*, SSu; *Staphylococcus sciuri*, SU; *Staphylococcus ureilyticus*, SW; *Staphylococcus warnerii*

Staphylococcus pettenkoferi was identified in oro-nasal and ulcer sites of one (1.3%) patient with a superficial ulcer (U1 [N and U sites]), *S. haemolyticus* was identified in both oro-nasal and ulcer sites of 2/76 (2.6%) patients (U17 [N and U sites], U72 [N, OC and U sites]), and *S. saprophyticus* was identified in both sites of 1/76 (1.3%) patients (U41 [N and U sites]) (Table 4.7).

4.4. Discussion

The aim of the present investigation was to investigate the prevalence of staphylococcal species in multiple anatomical sites of patients with type II diabetes both with and without DFUs, and to determine if the prevalence of distinct species differed between the patient cohorts. The study also sought to determine if the same staphylococcal species were present in oro-nasal and ulcer sites of patients with DFUs at the same point in time, to determine if the oro-nasal cavity could be a potential endogenous staphylococcal reservoir for DFUIs. In combination with these investigations, the periodontal health and oral healthcare habits of both patient cohorts were explored in the context of the staphylococcal populations resident in each.

Staphylococcus aureus is frequently isolated from the oro-nasal cavity and perioral region, and previous studies indicated that the nasal cavity should be considered a possible endogenous reservoir for dissemination to other anatomical sites (265). *Staphylococcus aureus* was previously recovered in higher frequencies in patients with DFUs than in patients without DFUs (186). These findings are consistent with the findings of the present study, which revealed that *S. aureus* was significantly more prevalent in all anatomical sites sampled ($P<0.05$) from patients with DFUs (N:36/76 [47.4%], OR:35/76 [46.1%], PP:13/54 [24.1%], F:10/76 [13.2%], T:11/76 [14.5%], U:40/76 [52.6%]) compared to patients without DFUs (N:26/76 [34.2%], OR:25/76 [32.9%], PP:9/76 [11.8%], F: 4/76 [5.3%], T:3/76 [3.9%]) (Table 4.6). Although data regarding incidence of feet-touching is limited, it is likely that patients with DFUs touch their feet more often than patients without DFUs. Patients with DFUs typically visit the hospital more frequently than individuals with diabetes without a DFU due to overall diabetes-related deterioration or DFU management (266, 267). As DFUIs are predominantly caused by *S. aureus* (268-270), this likely results in contamination of the hands during footwear removal and DFU management appointments. Although the prevalence of *S. aureus* was higher in patients with DFUs than those without DFUs, the predominant species recovered from oro-nasal sites of patients with DFUs was *S. epidermidis*, along with other CoNS (Table 4.6). Thus, it is possible that poor ulcer health is the predominant reason for increased *S. aureus* load in the anatomical sites of patients with DFUs, rather than poor oral health and hygiene practices.

Like *S. aureus, S. epidermidis* was also found to be more prevalent across all anatomical sites in patients with DFUs (N:45/76 [59.2%], OR:56/76 [73.6%], PP:34/54 [62.9%], F: 30/76 [39.5%], T:19/76 [25.0%], U:11/76 [14.5%]) compared to patients without DFUs, (N:39/76 [51.3%], OR:36/76 [47.4%], PP:37/76 [35.5%], F:17/76 [22.4%], T:16/76 [21.1%]) (Table 4.6), in agreement with previously reporting findings from this laboratory

(186). The prevalence of *S. epidermidis* was significantly higher in the oral cavity ($P<0.01$) of people with DFUs relative to those without DFUs (Table 4.6). Periodontal prevalence of *S. epidermidis* was also significantly higher than the prevalence of *S. aureus* in periodontal pockets of patients with DFUs ($P<0.01$). These findings support the findings of previous studies, which indicated that periodontal disease may be an important factor for growth and survival of *S. epidermidis* populations in the oral cavity (236). As *S. epidermidis* is more prevalent than *S. aureus* in the periodontal pockets and less prevalent than *S. aureus* in the nares and DFUs, nasal carriage is the more likely endogenous reservoir for DFUIs as has been previously suggested (186, 271-273). However, although prevalence of these species was higher in the nares than periodontal pockets, this does not rule out the potential for *S. aureus* and *S. epidermidis* present in periodontal pockets to be an endogenous reservoir for DFUIs.

O'Connor *et al.* (2018) previously reported that *S. epidermidis* was more prevalent and abundant in the oral cavity of patients with PD than orally healthy individuals (236). The same study also reported that *S. epidermidis* was almost four times more prevalent and 19 times more abundant than *S. aureus* (236). *Staphylococcus epidermidis* was also more prevalent in oro-nasal sites in patients with DFUs (70/76 [92.1%]) compared to *S. aureus* (60/76 [78.9%]), although this difference was not significant. Although PD was not definitively diagnosed in the present study, the pocket depths, plaque indices and BOP measurements suggest that PD is highly likely in both patients with and without DFUs (Table 4.2)

As well as oral carriage, *S. epidermidis* was also more prevalent in finger and toe sites of patients with DFUs (F: 30/76 [39.5%], T:19/76 [25.0%]) than *S. aureus* (F:10/76 [13.2%], T:11/76 [14.5%]). This finding was unsurprising, as *S. epidermidis* is ubiquitous on human skin, however the prevalence of *S. epidermidis* was significantly higher in the finger of patients with DFUs than *S. aureus* ($P<0.01$) (Table 4.6).

The staphylococcal prevalence data obtained in the present study is also in agreement with the previous research by O'Connor *et al.* (2018), which reported increased prevalence and abundance of staphylococci in the oral cavity of patients with PD (236). It is likely that the majority participants in the study had PD to some degree based on the comparable plaque indices, probing depths and BOP measurements in both groups. It is also possible that the increased prevalence and abundance of staphylococcal populations in patients with DFUs correlates with previously mentioned lack of consistent oral hygiene practices and attitudes to self-care, which can arguably also result in the formation of DFUs in certain

circumstances. These factors could be the cause of increased prevalence of these species in the oral cavity, and across all anatomical sites in patients with DFUs (Tables 4.5 and 4.6).

Comparably to the increased prevalence of *S. aureus* and *S. epidermidis* amongst patients with DFUs, the mean microbial bioburden was also consistently higher in all anatomical sites of patients with DFUs than in patients without DFUs (Table 4.4). The higher abundance of staphylococcal populations in oro-nasal sites amongst patients with DFUs may correlate with the self-reported irregularity of toothbrushing in this group, as patients with DFUs reported less frequent toothbrushing than patients without DFUs (Table 4.2). As such, the average plaque score observed amongst the patients with DFUs was higher (mean = 72, range 1-100 %) than that of non-DFU patients (mean = 61, range 1-100 %) (Table 4.2), however this difference was not significant (P<0.99363). This increased staphylococcal abundance is also likely influenced by the staphylococcal load identified in the ulcers of patients with DFUs. Unlike O'Connor *et al.* 2018, the abundance data was not species-specific, thus it was not possible to ascertain whether increased levels of *S. aureus* and *S. epidermidis* result in the increased abundance of staphylococcal species in patients with DFUs than those without DFUs.

Both *S. aureus* and *S. epidermidis* were co-isolated from multiple anatomical sites of patients with DFUs, often also alongside multiple other CoNS species (Table 4.6). Interestingly, *S. aureus* and *S. epidermidis* were not co-isolated from any of the 76 ulcers sampled, despite each of these two species often being co-isolated with other CoNS species (Table 4.6). This finding correlates with previous research by McManus *et al.* (2020). It has previously been suggested that *S. epidermidis* is a potent inhibitor of *S. aureus* growth (274), as *S. epidermidis* is known to activate innate immune signalling pathways in human keratinocytes which augment antimicrobial peptide-mediated killing of *S. aureus* (275). These findings suggest *S. aureus* may only thrive in the absence of *S. epidermidis* when competing for niche positions such as DFUs. O'Connor *et al.* (2018) also previously reported a lack of *S. aureus* and *S. epidermidis* co-isolation from periodontal pockets and subgingival sites, and suggested this is possibly due to *S. epidermidis* out-competing *S. aureus* in anaerobic environments (236). The present study contradicts these findings however, as co-isolation of *S. aureus* and *S. epidermidis* was observed from the periodontal pockets of 9/54 (16.7%) patients with DFUs and 5/54 (8.9%) patients without DFUs.

The presence of the same staphylococcal species in both the oro-nasal cavity and ulcer sites was detected in 38/76 (50.0%) patients with DFUs investigated. Of these 38 patients, 26 harboured *S. aureus* in both oro-nasal and ulcer sites. As *S. aureus* is the predominant causative agent associated with DFUIs (33, 244, 252), this data presents a cause

for increased awareness of potential trafficking between oro-nasal and ulcer sites. Ten additional patients harboured *S. epidermidis* in both sites and two patients harboured *S. haemolyticus* in both sites (Table 4.7). The presence of *S. epidermidis* in oro-nasal and DFUs presents cause for deeper investigation. Due to the propensity of this species to form biofilm and encode resistance determinants to multiple distinct classes of antibiotics, the presence of this species in DFUs should not be overlooked or dismissed as a simple contaminant. This species has been reported to be more strongly associated with ischaemic ulcers where blood flow is restricted (33), which can prove highly difficult to treat. The present study does not support this previous association of *S. epidermidis* with ischaemic ulcers, as *S. epidermidis* was recovered from 11 ulcers, of which the majority (10/11 [90.9%]) were neuropathic. The ubiquity of this species on human skin may indicate the oro-nasal cavity is not a sole reservoir for *S. epidermidis*-based DFUIs, however its association with PD and the higher incidence of PD in patients with diabetes with/without DFUs is noteworthy and warrants closer investigation.

The oral health and periodontal examinations of the participants in the present investigation revealed important oral clinical findings, which is mostly overlooked in these patient groups. Patients with DFUs reported less frequent toothbrushing, correlating with increased dental plaque scores and the prevalence and abundance of staphylococcal species. The oral health conditions of both patient groups were suboptimal, with indicators of PD identified in the majority of patients both with and without DFUs (Table 4.2). This is perhaps unsurprising, considering the paucity of oral health education routinely provided to patients with diabetes. Allen *et al.* reported that out of a group of 101 participants with diabetes, only 33% of patient participants were aware of the increased risk of PD development associated with diabetes (276). Furthermore, a study conducted by Ahern and Nunn investigated whether primary care physicians were aware of the bidirectional relationship between diabetes and PD. Of the 79 primary care physicians who completed the survey, nearly 90% of primary care physicians reported that they were aware of the link, but did not inform patients of such, or emphasize the importance of dental care in relation to diabetes management. Furthermore, 93% of physicians did not refer patients with diabetes to dentists, or advise them to routinely attend dental appointments as part of their diabetes management plan (277). Ahern *et al.* previously reported survey results which indicated that dentists from a variety of specialities believed that dentistry was isolated from other healthcare settings in Ireland, and they communicated that a need for oral health advocacy by members of the dental profession existed (277). Indeed, based on the oral and periodontal health conditions described amongst patients with and without DFUs in the present study, the sub-optimal oral

health routines undertaken by these patients and the increased staphylococcal abundance and prevalence amongst patients with DFUs, it is obvious that oral health is completely overlooked in the management of patients with diabetes in Ireland. To address this issue, it is essential that there is a closer holistic approach undertaken between dental and medical practitioners. This is required for knowledge regarding the importance of the oro-nasal cavity in systemic diseases in patients with diabetes, and to develop preventative care options that could potentially mitigate possible secondary infections. Such secondary infections could stem from the oro-nasal cavity as well as improving oral health itself. The improvement of oral health amongst people with diabetes would improve their quality of life and is an extremely important consideration in the context of the dietary and nutritional requirements of this patient group.

Chapter 5

Comparative population structure analysis and molecular characterisation of *S. aureus* and *S. epidermidis* from multiple anatomical sites of people with diabetes, with and without foot ulcers.

5.1. Introduction

5.1.1. Staphylococcal populations in people with type II diabetes

5.1.1.1. Staphylococcus aureus *populations in people with periodontal disease and diabetes*

Nucleotide sequence-based epidemiological studies have been used to characterise the *S. aureus* population structure and most prevalent STs and lineages in Europe (278-280) since the start of this century. Enright *et al.* (2000) investigated the most common clonal lineages of *S. aureus* in 155 isolates from community and hospital-acquired invasive disease in Oxford in 1997 and 1998 by MLST for the first time (280). They reported that the most common MRSA isolates belonged to ST36 and ST22, while MSSA populations were more diverse, belonging to ST30, ST12, ST8, ST39, ST1 and ST5 (280).

Previous research from this department in 2019 identified numerous MRSA and MSSA STs in hospital wards in Dublin, Ireland, and reported the most prevalent STs in that study were ST5, followed by ST22 and ST45 (85). Correspondingly, *spa* type t548 (associated with ST5) was the most frequently identified *spa* type, followed by t032 (associated with ST22) and t230 (associated with ST45) in Irish hospitals (85)

Previous studies reported an increased incidence of nasal carriage of *S. aureus* in individuals with diabetes in contrast to individuals without diabetes (281-285). Other studies compared the prevalence of MRSA in nursing homes between patients with and without diabetes and, despite the fact that all patients are frequently exposed to a healthcare setting, identified that MRSA prevalence was significantly higher in individuals with diabetes than in those without (142).

Despite the clinical significance of *S. aureus* in the aetiology of DFUs, previous studies on *S. aureus* populations in people with diabetes predominantly focused on the characterisation of MRSA (286-288) populations, as MRSA has previously been detected in 9.9% of DFUs investigated (288, 289).

One study investigated the population structure of *S. aureus* in DFUs of inpatients in an Algerian hospital (290). The majority of *S. aureus* isolates identified as MRSA (*N*=73) belonged to the Brazillian clone ST239 (*N*=60 [82.2%]). Ten isolates were identified as the European clone ST80 (13.7%), while the final three isolates belonged to an unknown clonal complex (CC). Regarding MSSA, isolates belonged to diverse CCs including CC1 (*N*=3), CC15 (*N*=3), CC121 (*N*=2), CC9 (*N*=1), CC54 (*N*=1) and ST152 (*N*=1).

A study by Dunyach-Remy *et al.* (2017) investigated the nares as a potential reservoir for DFUIs (98) by comparatively investigating nasal and ulcer isolates from individuals by DNA microarray profiling. This study investigated 276 patients with diabetes

and identified 101 patients (36.6%) with *S. aureus* in both nasal and DFU sites. Genetic analysis of the organisms at both sites of the 101 patients using the DNA microarray identified 65% of the participants harboured indistinguishable *S. aureus* strains at the nares and DFU based on array profiles, and reccommended nasal screening for *S. aureus* in people with diabetes and DFUs (98). Dunyach-Remy *et al.* (2017) reported that ST398 MSSA (*N*=26) was the most common lineage among isolates from DFUs and this specific clone was exclusively isolated from osteomyelitis cases from the same area in the patients investigated in this study (98).

Al-Bakri *et al.* (2021) characterised staphylococcal populations from the nares and DFUs in Jordinian patients by WGS and recovered *S. aureus* from the DFUs and nares of 14.2% and 9.8% of patients, respectively. Of the *S. aureus* isolates recovered, 93% of DFU isolates and 70% of nasal isolates were MRSA (291). Isolates were screened for SCC*mec* and *spa* type identification. SCC*mec* IV was the most prevalent SCC*mec* type identified, and t386 the most frequent *spa* type identified. The MRSA exhibited resistance to aminoglycosides, minoglycosides, fluoroquinolones and macrolides, and harboured the PVL toxin genes *lukF*-PV and *lukS*-PV, *tst*, *sea* and *hlg* virulence genes (291).

Another investigation conducted by Ferhaoui *et al.* (2023) characterised isolates of *S. aureus* (*N*=32) from the skin and DFUs of Algerian patients with DFUs by *spa* type and SCC*mec* type, and utilised MLST to caterogorise the isolates by ST, antimicrobial resistance and virulence genes (292). Among the 32 isolates of *S. aureus* investigated, 27 harboured *mecA*, 18 of which were recovered from DFUs. Isolates of *S. aureus* were identified as ST672, ST80, ST241, ST1, ST97, ST291 and four unknown STs (STNF). Eight *spa* types were identified, the most prevalent of which was t044 (13/32 [40.6%]), followed by t037 (9/32 [28.1%]), t3841 (3/32 [9.4%]), t1247 and t127 (2/32 each [6.3%]), t639, t9432 and t937 (1/32 each [3.1%]). Two *SCCmec* types were identified in MRSA isolates, predominantly SCC*mec* type IV (19/27 [70.4%]), and SCC*mec* type III (8/27 [29.6%]). Thirty-five virulence encoding genes were identified in the *S. aureus* population, including six haemolysins, 13 enterotoxins and 16 staphylococcal-like enterotoxins (292). Genes encoding *β*-lactam resistance, *mecA* and *blaZ*, were detected among *S. aureus* isolates at frequencies of 84.4% (27/32) and 50% (16/32), respectively.

The most well-known leukotoxin is the PVL toxin, a pore-forming toxin that confers cytotoxicity on neutrophils and phagocytic cells (42, 293). Strains of *S. aureus* which are PVL-positive are responsible for skin and soft tissue infections (SSTIs), aggressive bone and joint infections, and necrotizing pneumonia (290, 294, 295). A high prevalence of PVL was previously linked with community-associated MRSA (CA-MRSA) infections (296), and has

been identified in isolates ranging from 5-67% (296-299). The presence of PVL-positive *S. aureus* isolates in ulers indicates a poor prognosis, as this gene increases the severity of the infection in patients with DFUIs (300).

Despite the high prevalence of PD amongst people with diabetes, and the high prevalence of staphylococcal species in the oral cavities of people with PD, at the time of writing, no known studies to date have characterised oral and periodontal staphylococcal populations by molecular typing methods in people with diabetes.

Colombo *et al.* (2023) investigated antimicrobial resistance and virulence genes from staphylococci recovered from dental plaque from subgingival and periodontal sites from 142 orally healthy participants, 101 from individuals with gingivitis and 302 from patients with periodontal disease (301), although none of these participants had diabetes. Staphylococci were most frequently identified in the subgingival plaque of patients with periodontitis, followed by gingivitis and finally orally healthy participants. The predominant species recovered across all patient cohorts were *S. epidermidis* (59%) and *S. aureus* (22%). Twenty-two isolates were identified as methicillin resistant staphylococci, only five of which were identified as MRSA by disc diffusion assay, a clinical diagnostic tool determining resistance to agar plate-embedded antibiotics. However, *mecA* was detected by PCR identified in a further 25 staphylococcal isolates, predominantly *S. epidermidis* (*N*=14) and *S. aureus* (*N*=7). Genes encoding virulence factors were identified by PCR, with *groEL* (encoding heat shock chaperonin), *cna* (encoding collagen binding factors), and *ebpS* (encoding elastin binding proteins) being most prevalent and identified most frequently in isolates recovered from people with PD. The *lukF/lukS-pvl* positive strains of *S. aureus* were associated with deeper periodontal pockets and attachment loss (301). It is likely that the presence of *pvl* resulted in an increase in pathogenicity in these sites, causing a heightened immune response, and tissue destruction.

While *S. aureus* from subgingival and chronic wounds have been widely characterised, there are no studies comparing oral- and ulcer- *S. aureus* populations.

5.1.2.2 Staphylococcus epidermidis *populations in people with periodontal disease and diabetes*

The previously described Al-Bakri *et al.* (2021) study which characterised staphylococcal populations in the nares and DFUs in Jordinian patients with diabetes also reported that CoNS were the most common staphylococci recovered from DFUs (63.3%). These CoNS were predominantly identified as *S. epidermidis* in both the nares (39.2%) and DFUs (7.2%).

Previous investigations have identified *S. epidermidis* as a prominent coloniser of the oral cavity, periodontal and subgingival sites of individuals with periodontal disease and peri-implantitis (302). Many strains of *S. epidermidis* harbour variations of the arginine catabolic mobile element (ACME), a mobile genomic island that was first described in MRSA and is considered to enhance transmission, persistance and survival in anaerobic or environments lacking nutrients. Since its first description, ACME has been identified in multiple different species of staphylococci, including *S. epidermidis*, *S. capitis* and *S. haemolyticus*. There are three gene clusters which are prevalent in ACME, the *arc, opp* and *kdp* operons. The *arc* gene encodes a secondary arginine deiminase system. The *opp* gene encodes an oligopeptide permease ABC transporter. The *kdp* operon encodes a potassium ABC transporter (302). These genes are homologs of virulence determinants in other bacterial species (303). To date, six distinct ACME types have been described in *S. epidermidis* based on the presence of the *arc, opp3* and *kdp* operons. The ACME types described are defined as types I (*arc*+*opp3*), II (*arc*), III (*opp3*), IV (*arc*+*kdp*), V (*arc*+*opp3*+*kdp*), and VI (*kdp*) (302, 304). To date, only types I-III have been described in MRSA, whereas all six ACME types have been identified in *S. epidermidis* (52, 302, 305). O'Connor *et al.* identified ACME in 100/179 (55.8%) isolates of *S. epidermidis* recovered from periodontal and subgingival sites using multiplex PCR and DNA microarray profiling (302). This study identified five key ACME types (I, II, III, IV and V) amongst all isolates investigated, and reported that the presence of ACME was significantly higher among isolates from infected subgingival peri-implant sites than non-infected subgingival implant sites (P<0.01). It was also significantly more prevalent in isolates of *S. epidermidis* recovered from periodontal pockets than subgingival sites of periodontally healthy individuals (P<0.05).

However, despite a multitude of studies of molecular characterisation of *S. epidermidis* from bloodstream or indwelling medical devices infections, there is a lack of studies involving characterisation of *S. epidermidis* from chronic wounds and from people with diabetes. Many studies have described the role of *S. aureus* in chronic wounds, such as DFUs, including distinct STs and CCs, however, very few comparative investigations exist for *S. epidermidis*. One study investigated *S. epidermidis* and *S. aureus* populations in DFUs and healthy skin of Algerian patients (292). Among isolates of *S. epidermidis* investigated (N=10), 6/10 (60.0%) were identified as ST54, 2/10 (20.0%) as ST35 and 1/10 (10%) as ST640. Antimicrobial resistance genes detected in *S. epidermidis* included *fusB* and *fosB* genes, detected in 9/10 (90.0%) of isolates. In addition, *msr*(A) and *mph*(C) were detected in 2/10 (20.0%) *S. epidermidis* isolates and *erm*(C) was detected in 1/10 (10.0%) isolate. The

aph(3')-III/ *aph*(3')-IIIa (6/8 [75%]) and *tetK* (7/10 [70.0%]) genes were also identified in *S. epidermidis* (292). More comprehensive research is required to characterise strains from chronic DFUs in order to better understand the potential of this species as a causative pathogen in periodontal disease and ulcer infection amongst people with diabetes.

5.1.2. Study Aims

Whilst a vast expanse of research has undertaken molecular characterisation of *S. aureus* and *S. epidermidis*, there remains considerably less existing literature describing *S. aureus*, and in particular *S. epidermidis*, populations from chronic wounds with those in oral sites. To date, no other research groups have reported the investigatation of a possible microbial link between oral and ulcer sites. Previous investigations from this department reported that oral carriage of staphylocci is significantly higher in patients with periodontal disease, and preliminary investigations have reported the oral cavity as a possible endogenous reservoir for DFUIs utilising wgMLST (48, 99). It is entirely possible that the oral cavity is an endogenous microbial reservoir, as previous research implicated the nasal cavity as an endogenous reservoir for *S. aureus*-based DFUIs (98). Furthermore, investigations of the periodontal health of people with diabetes and characterisation of oral *S. aureus* and *S. epidermidis* populations in people with diabetes in Ireland are very limited. This study aimed to investigate if oral and periodontal isolates of both *S. aureus* and *S. epidermidis* are closely related to isolates recovered from other anatomical sites, particularly DFUs, in order to ascertain if the oro-nasal cavity is an endogenous reservoir for *S. aureus* and *S. epidermidis*-based DFUIs.

5.2. Materials and Methods

5.2.1. Selection of isolates for whole genome sequencing

Isolates of *S. aureus (N*=371) and *S. epidermidis (N*=327*)* from multiple anatomical sites of patients both with and without DFUs were subjected to WGS using the MiSeq desktop sequencer (Illumina), as previously described in Chapter 2, Section 2.9. Where possible, a minimum of two *S. aureus* isolates and four *S. epidermidis* isolates per patient sample site were selected for WGS. The *S. aureus* isolates subjected to WGS were recovered from 44 distinct patients with DFUs [OC: 56 N: 71, PP: 17, F: 13, T: 14, U: 95], and 28 patients without DFUs [OC: 48, N: 44, PP: 6, F: 4, T: 3]. The *S. epidermidis* isolates subjected to WGS were recovered from 36 distinct patients with DFUs [OC: 53, N: 51, PP: 55, F: 16, T: 17, U: 32], and 18 patients without DFUs [OC: 32, N: 42, PP: 15, F: 9, T: 5].

5.2.2. DNA extraction for molecular typing and whole genome sequencing

Genomic DNA was extracted for detection of ACME by multiplex PCR, *spa*-typing PCR and for WGS as previously described in Chapter 2, Sections 2.4.1 and 2.4.2.

5.2.3. Detection of ACME by Multiplex PCR

Definitively identified *S. epidermidis* isolates (*N*=297) were subjected to ACME detection using multiplex PCR incorporating the previously described oligonucleotide primers which target the *arc*, *opp3* and *kdp* operons associated with previously described ACME types I-VI (52, 54, 304, 305). Isolates subjected to ACME typing PCR were chosen to represent multiple anatomical sites from patients both with and without DFUs. Multiple isolates were taken per patient site due to the potential diversity of ACME in *S. epidermidis* populations.

Separate multiplex PCR amplifications were performed for each isolate using 30 ng of genomic DNA in 50 µl reaction volumes consisting of oligonucleotide primers at a concentration of 1 µM targeting the ACME-*arcA,* ACME-*opp3B* and ACME-*kdpA* (Table 5.1) genes, a 200 µM concentration of each deoxynucleoside triphosphate, 1.25 U of GoTaq polymerase (Promega) 10 µl (1×) of GoTaq FlexiBuffer (Promega) and 2.5 µM MgCl2. Cycling conditions consisted of 94°C for two min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s and followed by a final elongation step of 72°C for 10 min (236). The PCR products were separated by electrophoresis in 2% agarose gels and visualised as previously described in Chapter 2, Section 2.6.

Table 5.1. Oligonucleotide primers used for molecular typing techniques.

Primer	Amplicon size (bp)	Primer sequence (5'→ 3')	Primer target	Annealing Temperature (ºC)	Reference
*spa*F	Variable [a]	AGACGATCCTTCGGTGAGC	*S. aureus spa* gene	60	(306)
*spa*R		GCTTTTGCAATGTCATTTACTG			
ACME *arcA*F	724	CTAACACTGAACCCCAATG	ACME-*arcA*	60	(303)
ACME *arcA*R		GAGCCAGAAGTACGCGAG			
ACME *opp3B*F	530	GGATTCGCCCAAGTGATGACC	ACME-*opp3B*	60	(305)
ACME *opp3B*R		GACTGCTGGGTATGACGT			
ACME *kdpA*F	241	CGGTTTAACTGGTGCGTT	ACME-*kdpA*	60	(52)
ACME *kdpA*R		GCAATACATACAGCGTAGCC			

Abbreviation: bp, base pair.

[a]The polymorphic X region of the *spa* gene is highly diverse and consists of variable number of 24 bp repeats, which themselves can vary in size. Variation in this region is due to duplication, deletion, and mutation of repetitive units, and thus gives rise to variation in amplicon sizes (307).

5.2.4. Identification of *spa* types by PCR

Definitively identified *S. aureus* isolates (*N*= 267) were subjected to *spa* typing by PCR using previously described oligonucleotide primers (308). The X region of the *spa* gene, a variable region, underwent PCR amplification using primers and cycling conditions described by the European Network of Laboratories for Sequence Based Typing of Microbial Pathogens (SeqNet; http://www.seqnet.org). The PCR reaction volumes contained a final volume of 50 µl, containing1 µl DNA (50-100 ng/µl). Cycling for the *spa*-typing PCR includeD an initial denaturation at 94°C FOR 4 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 90 s, followed by a final extension at 72°C for 5 min. The amplicons were sequenced using Sanger Sequencing (Source Bioscience, Tramore, Wexford, Ireland). Analysis of *spa* sequences and *spa* type assignment was undertaken using the Ridom StaphType software package version 2.2.1 (Ridom GmbH).

5.2.5. Whole genome multilocus sequence typing for isolate relatedness

The isolate relatedness of *S. aureus* and *S. epidermidis* populations recovered from multiple anatomical sites of individual patients as well as the entire populations of each species recovered during the study was investigated via cgMLST and wgMLST as previously described in Chapter 2, Sections 2.9.2 and 2.9.3, respectively. Bioinformatic analysis for isolate relatedness was performed using the BioNumerics v8.0 (Applied Maths), and minimum spanning trees for visualising genetic relatedness were generated as described in section 2.9.5.

5.2.6. Detection of *spa* type, sequence types and staphylococcal antimicrobial and resistance genes based on WGS data

The *spa* types of *S. aureus* isolates were also determined or used to confirm previously PCR-identified *spa* types from assembled WGS datasets *in silico* via Ridom SeqSphere+ program v 7.0.4 as previously described in Chapter 2, Section 2.9.4. Sequence types (STs) of isolates were identified following *de novo* assemblies using the BioNumerics v8.0 software and incorporated SPAdes assembly software v3.7.1 (Applied Maths). The presence of antibiotic resistance and virulence factor encoding genes in both *S. aureus* and *S. epidermidis* datasets was detected using corresponding Ridom SeqSphere+ v7.0.4 task template tools as described in Chapter 2, Section 2

5.3. Results

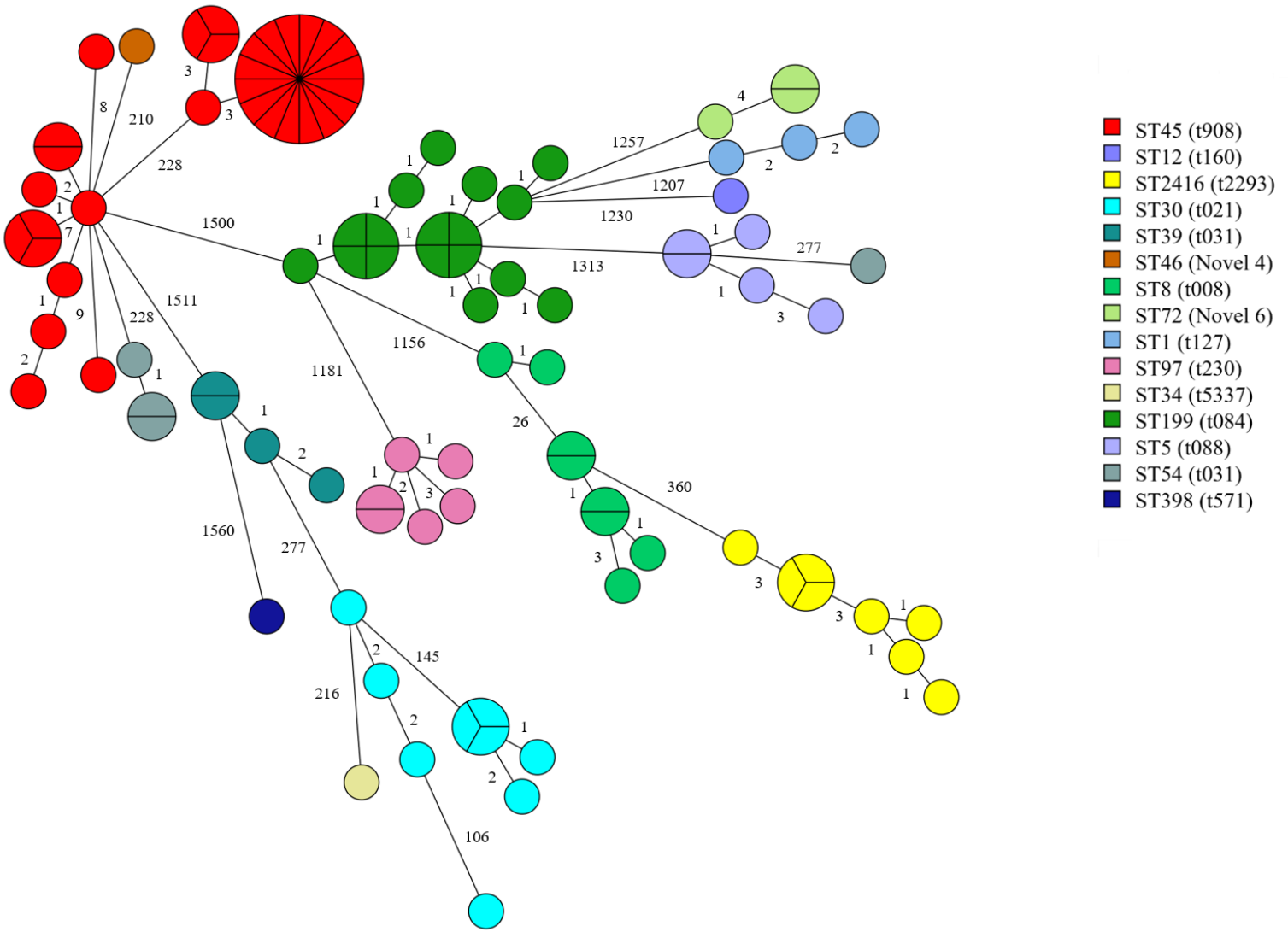
5.3.1. Investigation of *S. aureus* populations in people with diabetes

5.3.1.1. Sequence types and spa *types amongst S. aureus from patients without DFUs*

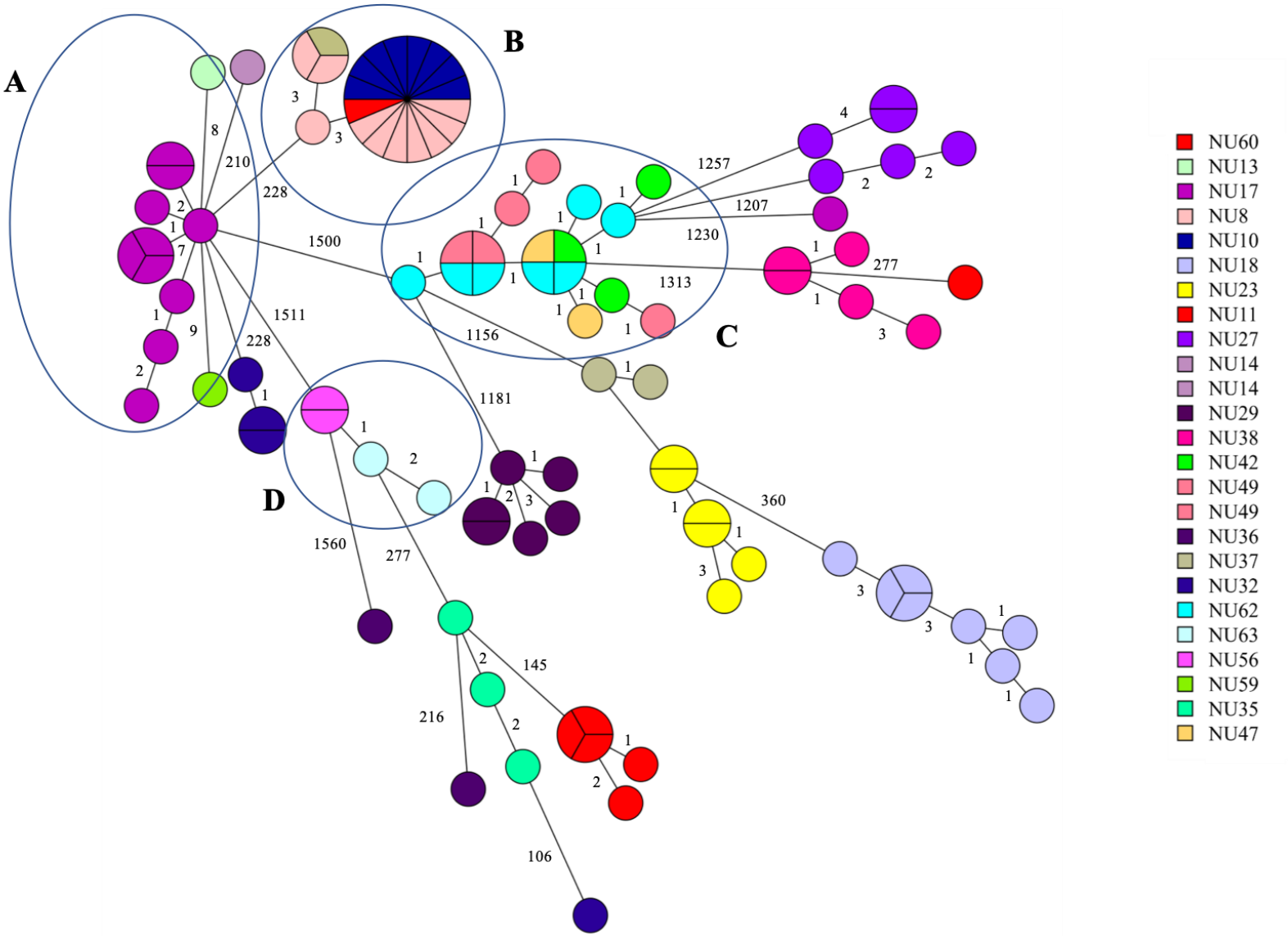
Staphylococcus aureus was recovered from 24/76 participants without DFUs. A total of 120 isolates, chosen as representatives of distinct participants and anatomical sites were investigated by *spa* typing (Table 5.2). The most prevalent *spa* type identified amongst these isolates was t908 (21/120 [17.5%] from four distinct participants), followed by t084 (22/120 [18.3%]) from three distinct participants), and t349 (19/120 [15.8%] from two distinct participants). The *spa* type t084 was most frequently identified in nasal isolates (11/49 [22.4%] from three participants), whereas t908 was most frequently identified from oral isolates (15/42 [35.7%] from three distinct participants) (Table 5.2).

A selection of 105 isolates, chosen as representatives of each participant, anatomical site and previously identified *spa* type underwent wgMLST. An MST was constructed based on the wgMLST datasets for these isolates to investigate the *S. aureus* population structure and predominant STs within this patient group (Fig. 5.1). The most frequently identified STs included ST45 (31/105 [29.5%] from six distinct participants) and ST199 (17/105 [16.1%] from three distinct participants). Isolates identified as ST45 belonged to *spa* types t908, t230 and t026 and were predominantly identified in isolates from the oral cavity (18/48 [37.5%] oral isolates from five distinct participants). Isolates identified as ST199 belonged to *spa* types t084 and t189 and were predominantly identified in isolates from the nasal cavity (10/48 [20.8%] isolates from five distinct participants) (Table 5.2 and Fig. 5.1). No MRSA isolates were detected in this patient population.

A second MST was constructed based on the same wgMLST dataset but indicating distinct participants rather than STs (Fig. 5.1, Panel B). This MST indicated that multiple *S. aureus* isolates recovered from different anatomical sites of the same individual were predominantly identified as the same ST, but also revealed the presence of four discrete clusters (A-D) of genetically identical isolates that were collected from multiple individuals (patients NU8, NU10, NU11, NU42, NU49, NU62).


ST45 (t908)
ST12 (t160)
ST2416 (t2293)
ST30 (t021)
ST39 (t031)
ST46 (Novel 4)
ST8 (t008)
ST72 (Novel 6)
ST1 (t127)
ST97 (t230)
ST34 (t5337)
ST199 (t084)
ST5 (t088)
ST54 (t031)
ST398 (t571)
1500
1511
1560
1181
1156
1313
1257
1207
1230
277
360
145
216
106
228
210

Panel A


A
B
C
D
NU60
NU13
NU17
NU8
NU10
NU18
NU23
NU11
NU27
NU14
NU14
NU29
NU38
NU42
NU49
NU49
NU36
NU37
NU32
NU62
NU63
NU56
NU59
NU35
NU47

Panel B

Figure 5.1. Minimum spanning trees (MSTs) based on whole genome multilocus sequence typing (wgMLST) analysis of 3,904 target genes for 105 *S. aureus* isolates from multiple anatomical sites (48 oral, 44 nasal, 6 periodontal pocket, 4 finger, 3 toe) of 28 distinct participants without DFUs. Each node (or portion of a node) represents one distinct isolate. The numbers aligned with each branch indicate the numbers of allelic differences between neighbouring isolates. Isolates that differ from each other by ≤24 allelic differences are considered closely related based on previously described relatedness thresholds (87, 309, 310) and are indicated by their enclosure within large blue circles. **Panel A:** MST indicating distinct sequence types (STs) according to colour, identified amongst isolates from multiple anatomical sites of 28 distinct participants. The coloured legend indicates STs and corresponding *spa* types associated with each ST, where applicable. Abbrevations: NU (Non-Ulcer); NT (Non Typable). **Panel B:** MST showing isolates recovered from distinct participants illustrated by corresponding colours as indicated in the legend along with patient numbers.

Table 5.2. Sequence types and *spa* types identified from *S. aureus* isolates recovered from patients with and without DFUs

Sample site	Isolates (*N*)	Patients (*N*)	Sequence type (*N*)	*spa* type (*N*)
			Patients without DFUs	
OR	48	13	ST199 (5), ST2416 (4), ST30 (8), ST45 (19), ST5 (3), ST72 (3), ST8 (3), ST97 (3)	t349 (4), t230 (3), t084 (6), t908 (15), t021 (7), t2293 (4), t088 (3)
NS	45	14	ST199 (10), ST2416 (4), ST30 (2), ST34 (4), ST398 (1), ST45 (12), ST46 (1), ST5 (2), ST54 (3), ST8 (3), ST97 (3)	t349 (6), t084 (11), t908 (3), t015 (1), t130 (1), t230 (8), t2293 (4), t031 (5), t571 (1), t088 (3), t399 (2), t002 (3), t180 (1)
PP	6	4	ST12 (1), ST199 (2), ST45 (1), ST8 (2)	t349 (8), t084 (5), t908 (3), t160 (1), t008 (2), t2947 (2), t189 (3)
F	3	2	ST34 (1), ST54 (1), ST97 (1)	t230 (1), t5727 (1), t5337 (1), t002 (2)
T	3	1	ST1 (3)	NA
			Patients with DFUs	
OR	56	14	ST45 (11), ST1 (6), ST12 (3), ST15 (6), ST22 (2), ST30 (8), ST34 (7), ST39 (3), ST398 (1), ST46 (3), ST5 (5), ST8347 (1)	t230 (18), t908 (7), t2886 (5), t2293 (2), t18906 (1), t021 (3), t571 (2), t084 (2), t190 (1), t127 (6), t067 (2), t233 (2), t015 (3), t088 (3)
NS	71	17	ST1 (10), ST12 (3), ST15 (6), ST22 (2), ST30 (16), ST34 (7), ST39 (9), ST398 (2), ST45 (2), ST46 (3), ST5 (6), ST59[a] (3), ST97 (2)	t2802 (4), t349 (4), t021 (5), t2866 (10), t363 (5), t20284 (1), t5337 (3), t084 (1), t127 (11), t548 (4), t571 (2), t223 (2), t015 (2), t4802 (2), t088 (3), t902 (1), t1451 (2), t008 (1)
PP	17	6	ST1 (1), ST46 (5), ST15 (4), ST22 (2), ST39 (3), ST45 (1), ST5 (1)	t908 (1), t2866 (4), t3132 (1), t084 (1), t088 (1)
F	13	5	ST46 (4), ST1 (2), ST12 (3), ST15 (3), ST22 (1)	t363 (1), t908 (1), t084 (2), t127 (2), t346 (2), t223 (1)
T	14	3	ST1 (6), ST22 (2), ST30 (2), ST45 (2), ST5 (1), ST8 (1)	t363 (6), t127 (6), t223 (1), t015 (1), t088 (2), t852 (1), t008 (1)
U	95	28	ST1 (21), ST12 (3), ST15 (10), ST22 (6), ST2416 (1), ST30 (7), ST34 (1), ST39 (7), ST398 (7), ST45 (5), ST5 (10), ST582 (3),	t2802 (4), t127 (20), t3132 (9), t363 (7), t18906 (4), t571 (2), t008 (4), t026 (3), t084 (2), t190 (3), t1451 (2), t548 (2), t346 (3), t166 (1), t548 (3), t067

	ST78 (5), ST8 (4), ST8347 (3), ST97 (2)	(2), t015 (2), t223 (2), t088 (3), t902 (2), t008 (1), t026 (1)

Abbreviations: OR; oral rinse, N; nares, PP; periodontal pocket, F; finger, T; toe, U; ulcer.
[a]Sequence types and *spa* types identified in methicillin-resistant *S. aureus* (MRSA) isolates (as well as in methicillin-susceptible *S. aureus*).

Isolates in cluster A were all identified as ST45, were separated from each other by an average of 2.5 allelic differences (range 1-9) and were recovered from three distinct partipants (NU13, NU17, NU59) who attended the sample collection site two weeks apart (NU13 and NU17) during 2020, and a further 44 weeks apart (NU59) during 2021. Isolates belonging to cluster B were also identified as ST45, were separated from each other by an average of 0.29 allelic differences (range 0-3) and were recovered from four distinct partipants (NU8, NU10, NU11 and NU37) who attended the sample collection site at a maximum of 44 weeks apart during 2020 and 2021. Isolates belonging to cluster C were all identified as ST199, were separated from each other by an average of 0.588 allelic differences (range 0-1) and were recovered from three distinct partipants (NU42, NU47, NU49 and NU62) who attended the sample collection site nine weeks apart during 2021. Isolates belonging to cluster D were all identified as ST39, were separated from each other by an average of 0.75 allelic differences (range 0-2) and were recovered from two distinct partipants (NU56 and NU63) who attended the sample collection site three weeks apart during 2021.

5.3.1.2 Sequence types and spa *types amongst* S. aureus *from patients with DFUs*

Isolates of *S. aureus* from multiple anatomical sites of 35/76 distinct participants with DFUs were investigated by *spa* typing (*N*=239) (Table 5.2). The most common *spa* types identified amongst isolates from this patient population were t127 (43/239 [13.6%] from eleven participants), t363 (19/239 [12.3%] from two participants), t230 (18/239 [10.2%] from four participants), t2866 (15/239 [10.2%] from one participants) and t088 (12/239 [8.2%] from one participant) (Table 5.2). The *spa* type t127 was predominantly identified isolates recovered from DFUs (*N*=20/43 [60.0%] ulcer isolates from five participants).

A selection of 266 isolates, chosen as representatives of each participant, anatomical site and previously identified *spa* type were also investigated by wgMLST to determine isolate relatedness within each participant and amongst this cohort in general (Table 5.2 and Fig. 5.2, Panel A). The most frequently identified STs identified from isolates recovered from this patient population included ST1 (46/266 [17.3%] from eight distinct

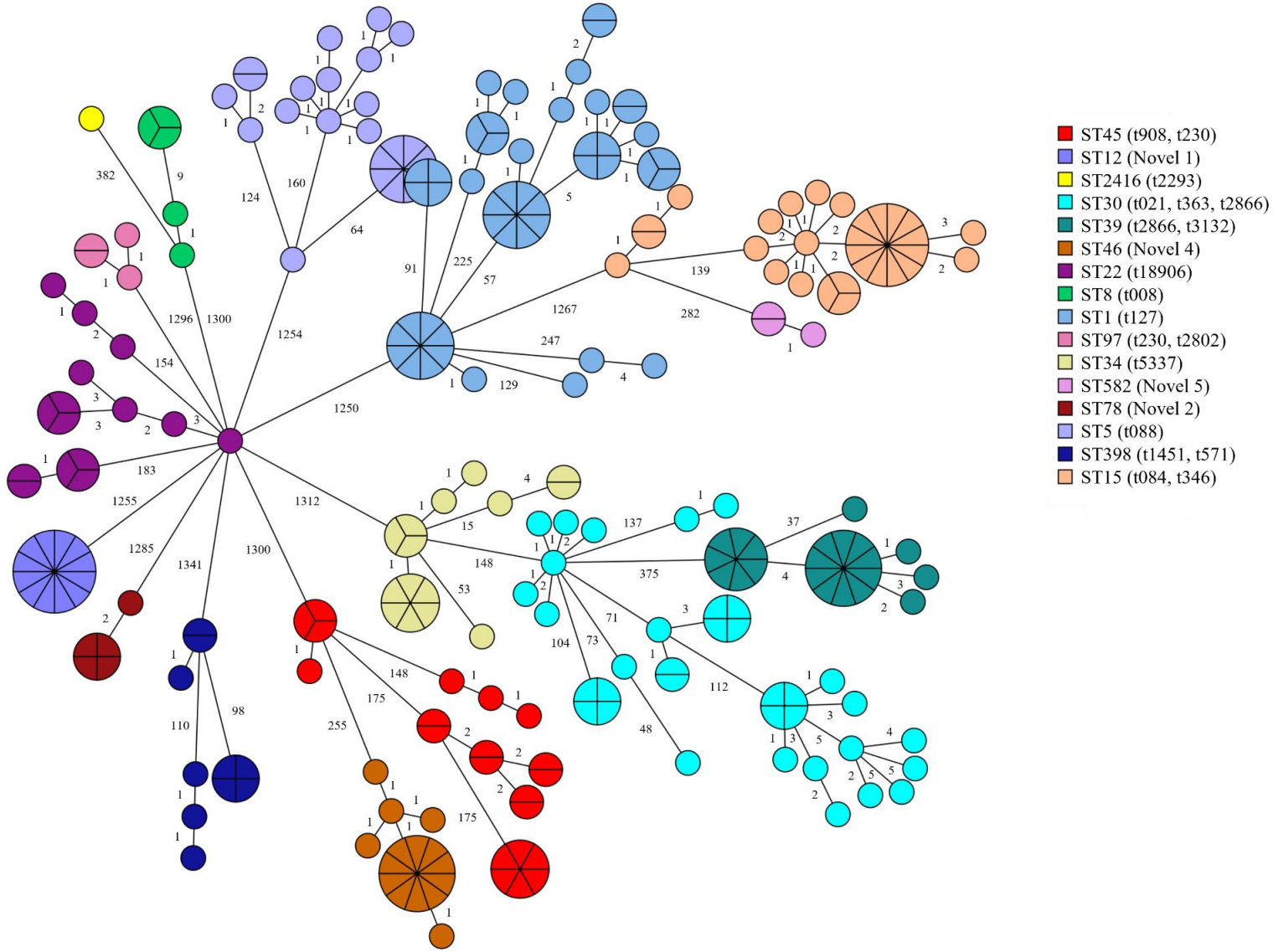
participants), ST30 (35/266 [13.2%] from six distinct participants) and ST15 (29/266 [10.9%] from four distinct participants).

Isolates identified as ST1 belonged to the *spa* type t127 and were most commonly collected from DFUs (*N*=21) of eight distinct patients. Isolates identified as ST30 belonged to the *spa* types t021, t363, t2866 and t363 and were most frequently recovered from the nares of this patient group (19/35 [54.3%] from five distinct patients).

Ulcer isolates comprised the majority of ST15 (10/29 [34.5%] from three participants) strains. The ST15 isolates belonged to *spa* types t084 and t346. ST39 isolates were predominantly identified amongst nasal isolates from one patient (*N*=8) and belonged to the *spa* types t2866 and t3132 (Table 5.2).

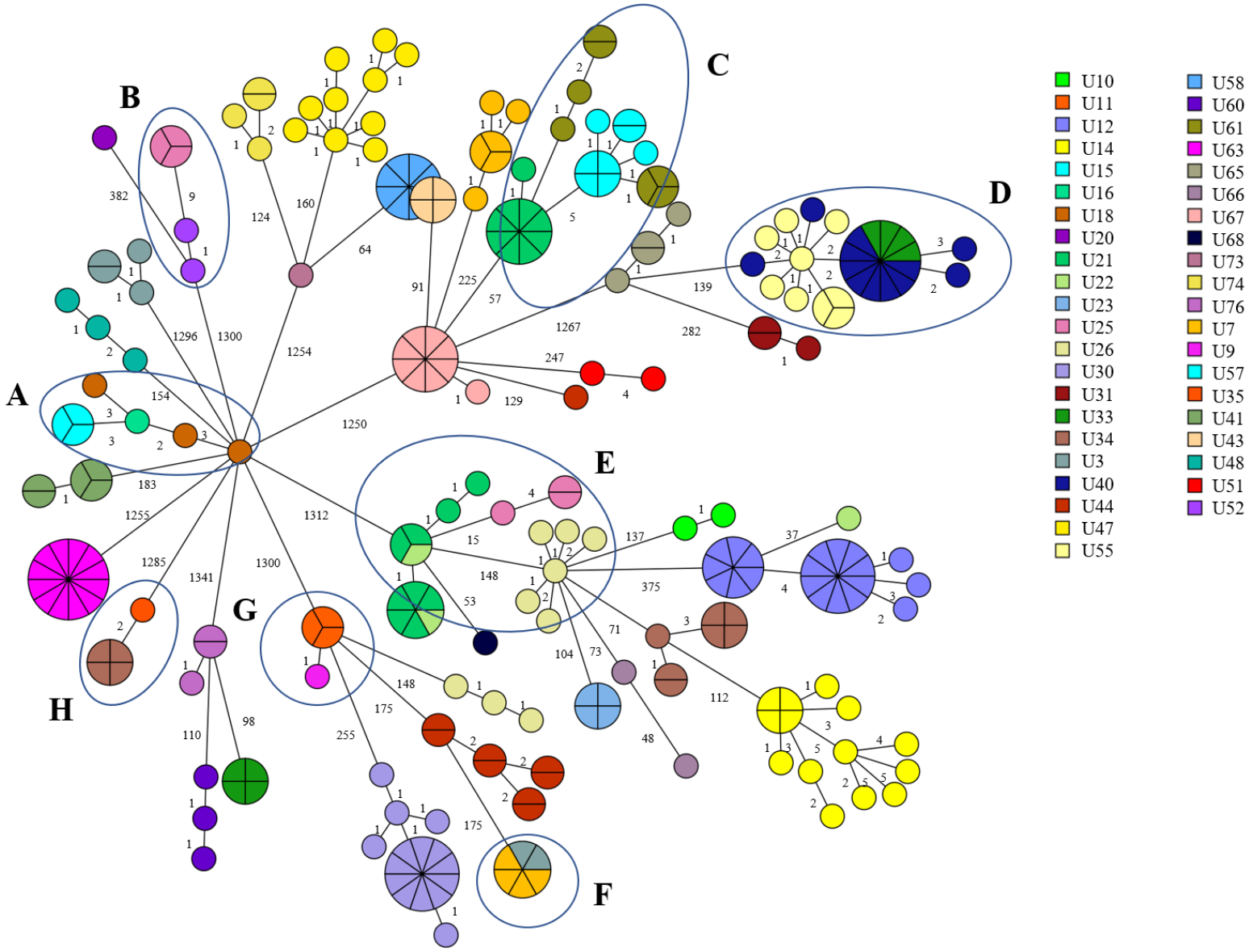
The second participant-orientated MST (Fig. 5.2, Panel B) indicated that multiple *S. aureus* isolates (*N*=175) recovered from different anatomical sites of the same individual were identified as the same ST, however, this MST also revealed the presence of eight distinct clusters (A-H) of highly related isolates from multiple distinct participants (patients U3, U7, U9, U11, U15, U16, U18, U21, U22, U25, U26, U33, U34, U35 U40, U52, U55, U57 and U61).

Isolates belonging to cluster A were identified as ST22, were separated from each other by an average of 1.57 allelic differences (range 0-3), and were recovered from three distinct participants (U15, U16 and U18), who attended the clinic six weeks apart during 2020. Isolates within cluster B were identified as ST8, were separated from one another by two allelic differences (range 0-9) and were recovered from two distinct participants (patients U25 and U52), who attended the clinic > 12 months apart between 2019-2021. Cluster C isolates were identified as ST1, were separated by an average of 1.25 allelic differences (range 0-17) and were recovered from three distinct participants (patients U21, U57 and U61) who attended the clinic 108 weeks apart between 2020 and 2023. Isolates within cluster D were identified as ST15 and were separated by an average of 0.6 allelic differences (range 0-3). These isolates were recovered from three distinct participants (patients U33, U40 and U55) who attended the clinic 50 weeks apart in 2021-2023.


ST45 (t908, t230)
ST12 (Novel 1)
ST2416 (t2293)
ST30 (t021, t363, t2866)
ST39 (t2866, t3132)
ST46 (Novel 4)
ST22 (t18906)
ST8 (t008)
ST1 (t127)
ST97 (t230, t2802)
ST34 (t5337)
ST582 (Novel 5)
ST78 (Novel 2)
ST5 (t088)
ST398 (t1451, t571)
ST15 (t084, t346)

Panel A

Panel B


A
B
C
D
E
F
G
H
U10
U11
U12
U14
U15
U16
U18
U20
U21
U22
U23
U25
U26
U30
U31
U33
U34
U3
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U61
U63
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U67
U68
U73
U74
U76
U7
U9
U57
U35
U41
U43
U48
U51
U52

Figure 5.2. Minimum spanning trees (MSTs) based on whole genome multilocus sequence typing (wgMLST) analysis of 3,904 target genes for 266 *S. aureus* isolates from multiple anatomical sites (56 oral, 71 nasal, 17 periodontal pocket, 13 finger, 14 toe and 95 ulcer) of 42 distinct participants with DFUs. Each node (or portion of a node) represents one distinct isolate. The numbers aligned with each branch indicate the numbers of allelic differences between neighbouring isolates. Isolates that differ from each other by ≤24 allelic differences are considered closely related as previously described (87, 309, 310), and are indicated by their enclosure within large blue circles. **Panel A:** Minimum spanning tree indicating distinct sequence types (STs) according to colour, identified amongst isolates from multiple anatomical sites of 42 distinct participants. The coloured legend indicates STs and corresponding *spa* types associated with each ST, where applicable. Abbrevations: U (Ulcer); NT (Non Typable). **Panel B:** Minimum spanning tree constructed as per the MST indicated in Panel A, with isolates recovered from distinct participants illustrated by corresponding colours as indicated in the legend along with patient numbers. Abbreviations: U; Ulcer, ST; Sequence type.

Isolates belonging to cluster E were identified as ST34 and were separated by an average of 5.35 allelic differences. Isolates belonging to this cluster were recovered from three distinct participants (patients U21, U22 and U26), who attended the clinic 26 weeks apart between 2020 and 2021. Cluster F isolates, identified as ST45, exhibited zero allelic differences and were all genetically indistinguishable. Isolates within this cluster were recovered from two individual participants (U3 and U7), who attended the clinic three weeks apart in 2020. Isolates belonging to cluster G were identified as ST78 and were separated by an average of 0.4 allelic differences (range 0-2). Isolates within this cluster were recovered from two distinct participants (patients U34 and U35) who attended the clinic two days apart in 2022. Isolates within cluster H were also identified as ST45 and were separated by an average of 0.25 allelic differences (range 0-1). Isolates within this cluster were recovered from two distinct participants (patients U9 and U11) who attended the clinic 16 days apart in 2020.

Three *S. aureus* isolates subjected to WGS were subsequently identified as methicillin-resistant *S. aureus* (MRSA) following the detection of the *mecA* gene in the WGS datasets of the isolates collected from patients with DFUs, and were confirmed as methicillin resistant phenotypically using MRSA*Select*™ media. These isolates were recovered from the nares (*N*=3) of patient U11 and identified as ST59 (Table 5.2).

Multiple STs (ST1, ST2416, ST30, ST34, ST45, ST46, ST5, ST8 and ST97) were identified in participants without and with DFUs (Table 5.2). Isolates identified as ST45 were predominantly recovered from the oral cavity between both patient cohorts (*N*=24 from four participants). Isolates identified as ST30 (*N*=20, from six participants), ST34 (*N*=11 from three participants) and ST97 (*N*=5 from two participants) were predominantly recovered from the nares and, ST1 (*N*=15 from seven participants) and ST5 (*N*=7 from four participants) isolates were predominantly recovered from ulcers (Fig. 5.2).

5.3.1.3. Genes encoding antibiotic resistance determinants in S. aureus *isolates from patients without and with DFUs.*

The prevalence of genes encoding antibiotic resistance determinants and virulence factors was investigated amongst the *S. aureus* isolates subjected to WGS (*N* = 371) from patients without (*N*=24) and with DFUs (*N*=42). Eleven distinct antimicrobial resistance genes were detected amongst the 371 isolates investigated; *bla*Z, *erm*(C), *fosB*, *fusB*, *fusC*, *erm*(C), *mph*(C), *msr*(A), *qacA*, *tetK* and *mecA*, however, the majority of resistance genes were detected in isolates from patients with DFUs. The *blaZ*, *fosB* and *mph*(C) genes were detected in isolates from patients without DFUs but were less prevalent when compared with isolates recovered from patients with DFUs (Table 5.3).

The *blaZ* gene, encoding resistance to beta-lactams, was the most common resistance gene identified in 30/105 (28.5%) isolates from 4/24 (16.6%) patients without DFUs. The *blaZ* gene was identified in isolates investigated (148/266 [55.6%]) from 33 patients with DFUs. This difference was significant ($P<0.01$).

The *fosB* gene (encoding metallothiol transferase) was more prevalent in isolates recovered from patients with DFUs (67/266 [25.1%] from 15 participants) than participants without DFUs (12/105 [11.4%] from two participants). This difference was significant ($P<0.01$).

The fuscidic acid-encoding genes *fusB* (13/219 [6.3%] from eight participants) and *fusC* (29/219 [13.2%] from eight participants) were only identified in isolates recovered from patients with DFUs (Table 5.3).

The macrolide resistance encoding genes (311) *mph*(C), *msr*(A) and *erm*(C) were only identified from *S. aureus* isolates recovered from patients with DFUs, detected in 7/266 [2.6%] from two participants, 4/266 [1.5%] from two participants) and 8/266 [3.0%] from three participants, respectively.

The *qacA* gene, facilitating resistance to quaternary ammonium compounds (QACs) (312), was identified only in isolates recovered from patients with DFUs (14/266 [5.2%] from three participants) (Table 5.3).

Table 5.3. Antimicrobial agent resistance and virulence genes detected in *S. aureus* isolates from patients with and without DFUs

Sample Site	Patients (*N*)	Resistance Genes (*N*)	Virulence Genes (*N*)
		Patients without DFUs	
OR	5	*blaZ* (25), *fosB* (8)	*sec* (15), *lukD* (3), *hlgA* (24), *hlIII* (24), *splB* (3), *sspA* (24)
N	3	*blaZ* (4), *fosB* (1), *mph*(C) (1)	*sec* (3), *lukD* (2), *hlgA* (5), *hlIII* (5), *splB* (2), *sspA* (5)
PP	3	*blaZ* (1), *fosB* (3)	*sec* (2), *lukD* (3), *hlgA* (5), *hlIII* (5), *splB* (3), *sspA* (5)
F	0	NA	NA
T	0	NA	NA
		Patients with DFUs	
OR	14	*blaZ* (37), *fosB* (17), *fusB* (1), *fusC* (6), *mph*(C) (1), *msr*(A) (1), *qacA* (4), *erm*(C) (2)	*egc* (8), *sec* (12), *seh* (6), *hld* (8), *lukD* (9), *lukF/S* (6), *lukX/Y* (5), *hlgA* (38), *hlIII* (38), *splA* (16), *splB* (17), *sspA* (25), *tst* (2)
N	14	*blaZ* (28), *fosB* (16), *fusB* (5), *fusC* (7), *qacA* (5), *tetK* (2), *erm*(C) (2), *mecA* (3)	*seh* (5), *lukD* (18), *lukF/S* (12), *hlgA* (27), *hla* (3), *hlb* (6), *hld* (15), *hlIII* (19), *splA* (21), *splB* (23), *splE* (1), *sspA* (23), *tst* (2), *egc* (4), *lukX/Y* (9)
PP	4	*blaZ* (4), *fosB* (2)	*egc* (1), *sec* (1), *lukD* (2), *lukF/S* (1), *hlgA* (3), *hld* (1), *hlIII* (3), *splA* (2), *splB* (2), *sspA* (3)
F	7	*blaZ* (12), *fosB* (7), *fusB* (3), *fusC* (1)	*egc* (1), *seh* (1), *lukD* (5), *hlgA* (8), *hld* (1), *hlIII* (8), *lukX/Y* (1), *splA* (8), *splB* (8), *sspA* (5), *tst* (1)
T	7	*blaZ* (14), *fosB* (3), *fusB* (1), *fusC* (3), *mph*(C) (1), *msr*(A) (1), *tetK* (2)	*egc* (5), *seh* (2), *lukD* (2), *hlgA* (2), *lukF/S* (5), *hla* (1), *hld* (7), *hlIII* (7), *splA* (6), *splB* (6), *sspA* (7), *tst* (1)
U	28	*blaZ* (53), *erm*(C) (4), *fosB* (22), *fusB* (3), *fusC* (12), *mph*(C) (5), *msr*(A) (2), *qacA* (5), *tetK* (2)	*egc* (8), *seh* (8), *lukD* (28), *luk*(E) (9), *lukF/S* (11), *lukX/Y* (8), *hld* (12), *hlgA* (33), *hlIII* (44), *splA* (31), *splB* (26), *sspA* (31), *tst* (2)

Abbreviations: OR; Oral rinse, N; nares, PP; periodontal pocket, F; finger, T; toe, U; ulcer.

5.3.1.3. Genes encoding virulence factors in S. aureus *isolates from patients without and with DFUs.*

The prevalence of virulence factor-encoding genes was also investigated amongst the *S. aureus* isolates subjected to WGS. Fifteen distinct virulence factor determining genes were detected amongst the isolates recovered from both patient groups (*egc, sec, seh, hla, hlb, hld, lukD, lukF/S, lukX/Y, hlgA, hlIII, splA, splB, ssp*A, *tst*)). From this collection of identified virulence genes, eight were detected within isolates from both patient groups (*sec, lukD, hlgA, hlIII, splB, sspA*) (Table 5.3).

The superantigen staphylococcal enterotoxin C (*sec*), important for *S. aureus* associated infective endocarditis (313), was identified in isolates from both patients with (13/266 [4.8%] from five participants) and without (20/105 [19.0%] from four participants) DFUs. It was significantly more prevalent in isolates recovered from patients without DFUs than patients with DFUs ($P<0.01$).

The leukocidin encoding gene *lukD* was detected in isolates from both participant groups but was significantly more prevalent ($P<0.01$) in isolates recovered from patients with DFUs (64/266 [24.0%] from 15 participants) in contrast to patients without DFUs (8/105 [7.6%] from one participant).

The virulence gene *hlgA*, a haemolysin which plays a role in septic arthritis (314) was more prevalent among isolates recovered from patients with DFUs (111/266 [41.7%] from 24 participants) than in patients without DFUs (34/105 [33.6%] from four participants). This difference was not significant. Another haemolysin gene, *hlIII*, was detected in both participant groups. It was significantly more prevalent ($P<0.05$) in isolates recovered from patients with DFUs (119/266 [44.7%] from 25 participants) than in patients without DFUs (34/105 [32.3%] from four participants).

Genes encoding invasive toxins including *splB* and *sspA* were identified in both patient groups. The *splB* gene was significantly more prevalent in isolates from patients with DFUs (82/266 [37.4%] from 14 participants) than patients without DFUs (8/105 [7.6%] from one participants) ($P<0.01$).

The *sspA* gene was more prevalent in isolates recovered from patients with DFUs (94/266 [35.3%] from 15 participants) than in patients without DFUs (34/105 [32.3%] from four participants) (Table 5.3). However, this difference was not significant.

The *lukF/S-pvl* gene was only identified in isolates ($N=35$) recovered from nine participants with DFUs (Table 5.3). It was most prevalent in isolates from the nares of this

patient cohort (12/188 [6.3%], from six participants). It was also recovered from the DFUs of this participant cohort in very low prevalence (11/251 [4.3%], from five participants).

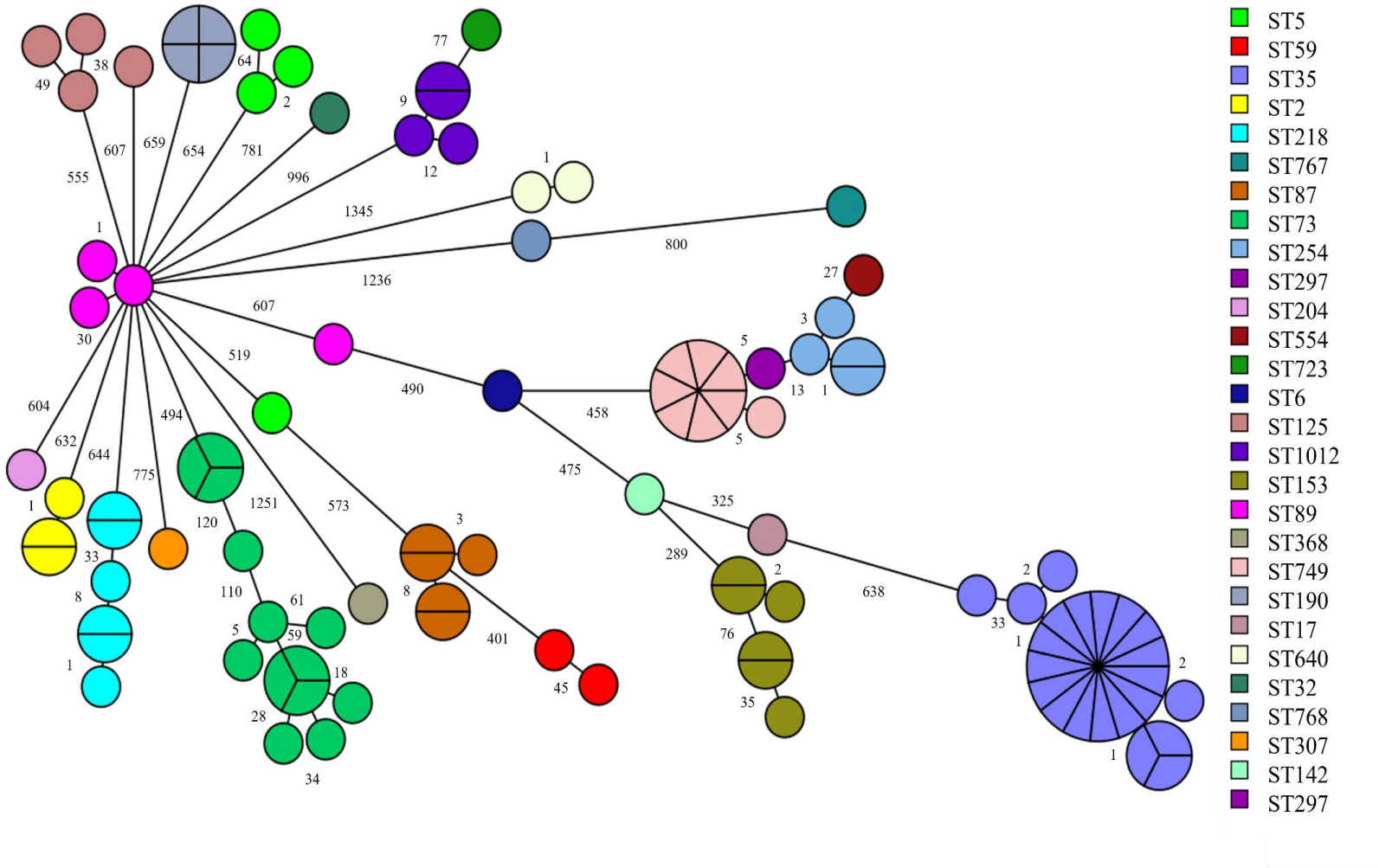
5.3.2. Investigation of *S. epidermidis* populations in people with diabetes

5.3.2.1. Sequence types identified in S. epidermidis *from people without DFUs*

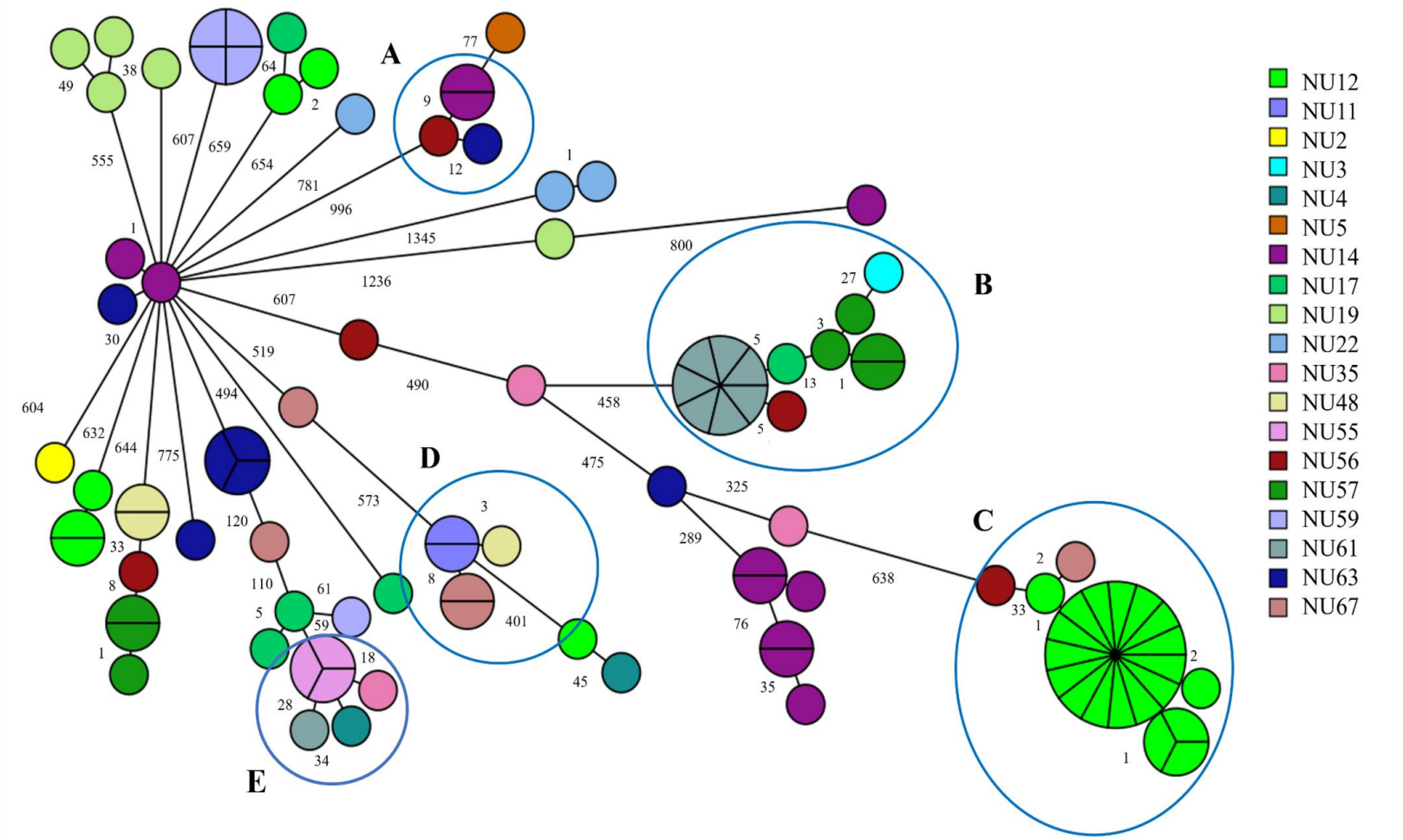
A collection of *S. epidermidis* isolates were chosen as representatives of multiple anatomical sites from 19 participants without DFUs and subjected to wgMLST (*N*=103), to determine isolate relatedness within each participant and amongst this cohort (Table 5.4). In contrast to the *S. aureus* populations from both patient groups, multiple *S. epidermidis* isolates collected from the same participant were identified as distinct STs. The presence of multiple, distinct *S. epidermidis* STs in the same individual was observed in 14 patients without DFUs (patients NU4, NU12, NU14, NU17, NU19, NU22, NU35, NU48 NU56, NU57, NU59, NU61, NU63, NU67) and 27 patients with DFUs (patients U1, U3, U4, U5, U9, U11, U12, U14, U15, U16, U18, U20, U22, U23, U24, U26, U31, U42, U45, U46, U49, U50, U51, U52, U53, U55 and U58) (Figs. 5.3 and 5.4).

The most frequently identified STs from isolates within this population were ST35 (23/103 [22.3%] from three participants), ST73 (17/103 [16.5%] from eight participants), ST153 (6/103 [5.8%] from one participant), and ST218 (6/103 [5.8%] from three participants). Sequence type 35 was most commonly identified in oral and (7/22 [31.8%]) and periodontal isolates (7/22 [31.8%%]) from one patient, and from the oro-nasal cavity of two other patients. Isolates identified as ST73 were recovered from the oro-nasal cavity of 8/19 (42.1%) distinct patients, which may indicate enrichment this ST in the oro-nasal cavity of people with diabetes. *Staphylococcus epidermidis* ST218 isolates were recovered from three individual patients and was predominantly identified in oral isolates (Table 5.4).

Ten *S. epidermidis* isolates from three participants without DFUs harboured the *mecA* gene which were identified as ST2 (*N*=5 recovered from periodontal, finger and toe isolates), ST5 (*N*=2, from nasal and periodontal isolates), ST7 (*N*=1, from an isolate recovered from the periodontal pocket), ST59 (*N*=1, from an isolate recovered from the periodontal pocket), and ST640 (*N*=2, from toe isolates).


ST5
ST59
ST35
ST2
ST218
ST767
ST87
ST73
ST254
ST297
ST204
ST554
ST723
ST6
ST125
ST1012
ST153
ST89
ST368
ST749
ST190
ST17
ST640
ST32
ST768
ST307
ST142
ST297

Panel A


A
B
C
D
E
NU12
NU11
NU2
NU3
NU4
NU5
NU14
NU17
NU19
NU22
NU35
NU48
NU55
NU56
NU57
NU59
NU61
NU63
NU67

Panel B

Figure 5.3. Minimum spanning trees (MSTs) based on whole genome multilocus sequencing typing (wgMLST) analysis of 4877 target genes for 103 *S. epidermidis* isolates from multiple anatomical sites (32 oral, 42 nasal, 15 periodontal pocket, 9 fingers, 5 toe) of 18 distinct participants without DFUs investigated by wgMLST. Each node (or portion of a node) represents one distinct isolate. The numbers aligned with each branch indicate the number of allelic differences between neighbouring isolates. Isolates that differ from each other by ≤24 allelic differences are considered closely related based on previously described relatedness thresholds (129, 315) and are indicated by their enclosure within large blue circles. MST indicating distinct sequence types (STs) according to colour, identified amongst isolates from multiple anatomical sites of 18 distinct participants. The coloured legend indicates STs identified in the isolate population. **Panel B:** MST showing isolates recovered from distinct participants illustrated by corresponding colours as indicated in the legend along with patient numbers. Abbreviations: NU; Non-Ulcer.

Table 5.4. Sequence types identified amongst *S. epidermidis* isolates recovered from patients without and with DFUs

Sample Site	Isolates *(N)*	Participants (*N*)	Sequence Types *(N)*
			Patients without DFUs
OR	32	14	ST1012 (2), ST125 (3), ST153 (2), ST218 (3), ST307 (1), ST35 (7), ST5 (2), ST73 (5), ST749 (3), ST87 (4),
NS	42	15	ST1012 (2), ST125 (1), ST154 (3), ST17 (1), ST190 (4), ST218 (3), ST254 (3), ST32 (1), ST35 (6), ST5 (1), ST6 (1), ST723 (1), ST73 (5), ST749 (5), ST87 (1), ST89 (4)
PP	15	5	ST125 (1), ST2 (1), ST142 (1), ST254 (1), ST35 (7), ST5 (1), ST59 (1), ST73 (1), ST89 (1)
F	9	4	ST130 (1), ST14 (1), ST153 (2), ST173 (1), ST2 (2), ST204 (1), ST297 (1), ST35 (2), ST368 (1), ST5 (1), ST155 (1), ST59 (1), ST73 (2), ST767 (1),
T	5	3	ST125 (1), ST554 (1), ST640 (2), ST768 (1)
			Patients with DFUs
OR	53	19	ST14 (1), ST2 (2), ST20 (1), ST204 (2), ST208 (2), ST22 (1), ST291 (1), ST297 (4), ST35 (10), ST384 (2), ST5 (5), ST554 (1), ST57 (2), ST59 (1), ST7 (1), ST73 (2), ST783 (2), ST83 (1), ST85 (2), ST86 (1), ST87 (3), ST88 (1), ST89 (4)
NS	52	15	ST1012 (1), ST130 (1), ST14 (1), ST173 (1), ST194 (2), ST2 (3), ST284 (1), ST297 (1), ST329 (5), ST35 (2), ST357 (2), ST5 (8), ST57 (3), ST59 (2), ST719 (3), ST723 (1), ST83 (3), ST85 (3), ST87 (8), ST88 (1), ST89 (2)
PP	55	17	ST125 (1), ST2 (8), ST204 (2), ST22 (3), ST278 (2), ST32 (1), ST35 (2), ST48 (1), ST5 (11), ST554 (1), ST567 (1), ST723 (1), ST73 (6), ST83 (3), ST85 (2), ST87 (2), ST88 (2), ST89 (1), ST915 (1)
F	16	4	ST130 (1), ST14 (1), ST153 (2), ST173 (1), ST204 (1), ST22 (1), ST329 (2), ST5 (4), ST59 (2), ST723 (1),
T	17	7	ST1012 (2), ST125 (1), ST329 (1), ST378 (1), ST6 (1), ST679 (1), ST765 (2), ST767 (5), ST87 (2),
U	31	10	ST2 (8), ST204 (1), ST212 (3), ST297 (2), ST5 (5), ST640 (1), ST73 (2), ST83 (1), ST87 (3)

Abbreviations: OR; Oral Rinse, N; Nares, PP; Periodontal Pockets, F; Finger, T; Toe, U; Ulcer.

5.3.2.2. Sequence types identified in S. epidermidis *from patients with DFUs*

A selection of 224 *S. epidermidis* isolates, chosen as representatives of distinct participants (*N*=32) and anatomical site underwent wgMLST to investigate isolate relatedness within each participant and amongst this cohort of patients with DFUs in general, and comprised 48 distinct STs (Fig. 5.4 and Table 5.4). The most common STs identified in this *S. epidermidis* population included ST5 (33/224 [14.7%] from nine participants), ST2 (20/224 [8.9%] from four participants), ST87 (17/224 [7.6%] from two participants) and ST35 (14/224 [6.3%] from five participants).

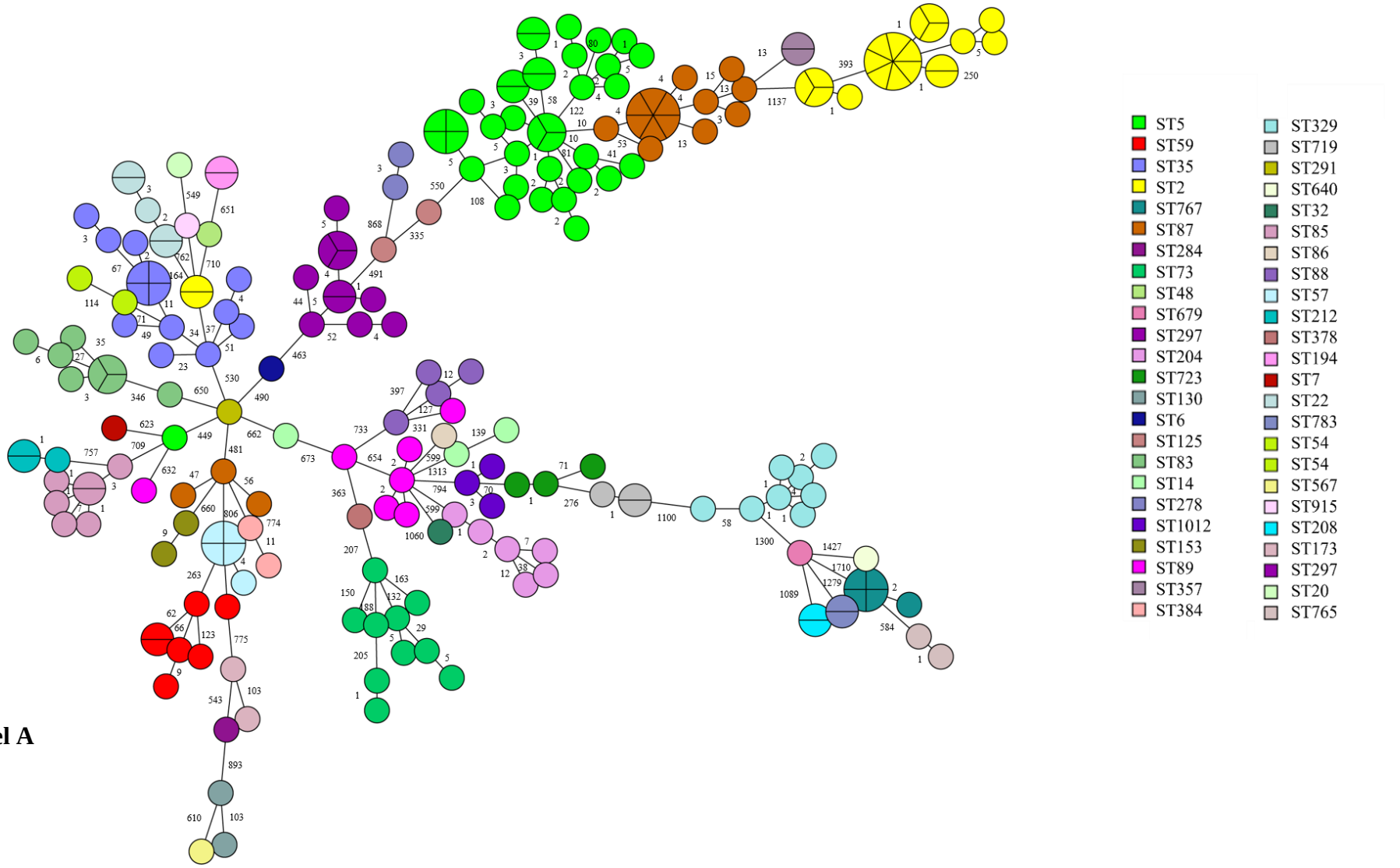
Isolates from nine separate patients were identified as ST5 and were most frequently detected among isolates recovered from periodontal pockets (11/33 [33.3%] from three participants). Isolates confirmed as ST2 were recovered equally from periodontal pockets (8/20 [40.0%]) and ulcer sites (8/20 [40.0%]) from two patients with DFUs. Isolates of *S. epidermidis* identified as ST87 were recovered from four participants from this cohort, most commonly from the nares (7/17 [41.1%] from four participants). Sequence type 35 was identified in oral isolates from three distinct patients and was recovered from isolates from multiple anatomical sites from five distinct patients. This ST was recovered in the lowest frequency among isolates recovered from both the nares and periodontal pockets from two patients in this population.

Twelve *S. epidermidis* isolates from three distinct participants with DFUs were identified as methicillin-resistant *S. epidermidis* (MRSE) following the detection of the *mecA* gene in the WGS datasets. These isolates were recovered from the oral cavity (*N*=4, identified as ST35 and ST291) and periodontal pockets (*N*=2, identified as ST35) from one patient, the nares (*N*=2, identified as ST5) from a second distinct participant and the toe (*N*=2, identified as ST765) and periodontal pockets (*N*=2, identified as ST2) from a final patient.

Five clusters (Clusters A-E; Fig. 5.4, Panel B) of closely related isolates recovered from distinct patients were identified. Isolates within cluster A, identified as ST87, were separated by an average of 5.1 allelic differences (range 0-15), and were recovered from two patients who attended the clinic 28 weeks apart in 2020. Isolates belonging to cluster B, identified as ST5, were separated by an average of 2.2 allelic differences (range 0-10), and were recovered from two patients who attended the clinic 52 weeks apart between 2019 and 2020. Isolates belonging to cluster C, identified as ST22, and were separated by an average of one allelic difference (range 0-2). These isolates were recovered from three participants who attended the clinic two weeks apart in 2019. Isolates belonging to cluster D, identified

as ST35, were separated by an average of 2.3 allelic differences (range 0-10), and were recovered from two patients who attended the clinic 27 weeks apart in 2020. Lastly, isolates belonging to cluster E were identified as ST204. These isolates were separated by an average of 4.6 allelic differences (range 2-7) and were recovered from two patients who attended the clinic 58 weeks apart in 2019 and 2020 (Fig. 5.4).

Fifteen STs were identified amongst *S. epidermidis* isolates from patients with and without DFUs (ST1012, ST125, ST153, ST2, ST297, ST32, ST35, ST5, ST59, ST6, ST640, ST73, ST767, ST87 and ST89) (Table 5.4).


ST5
ST59
ST35
ST2
ST767
ST87
ST284
ST73
ST48
ST679
ST297
ST204
ST723
ST130
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ST125
ST83
ST14
ST278
ST1012
ST153
ST89
ST357
ST384
ST329
ST719
ST291
ST640
ST32
ST85
ST86
ST88
ST57
ST212
ST378
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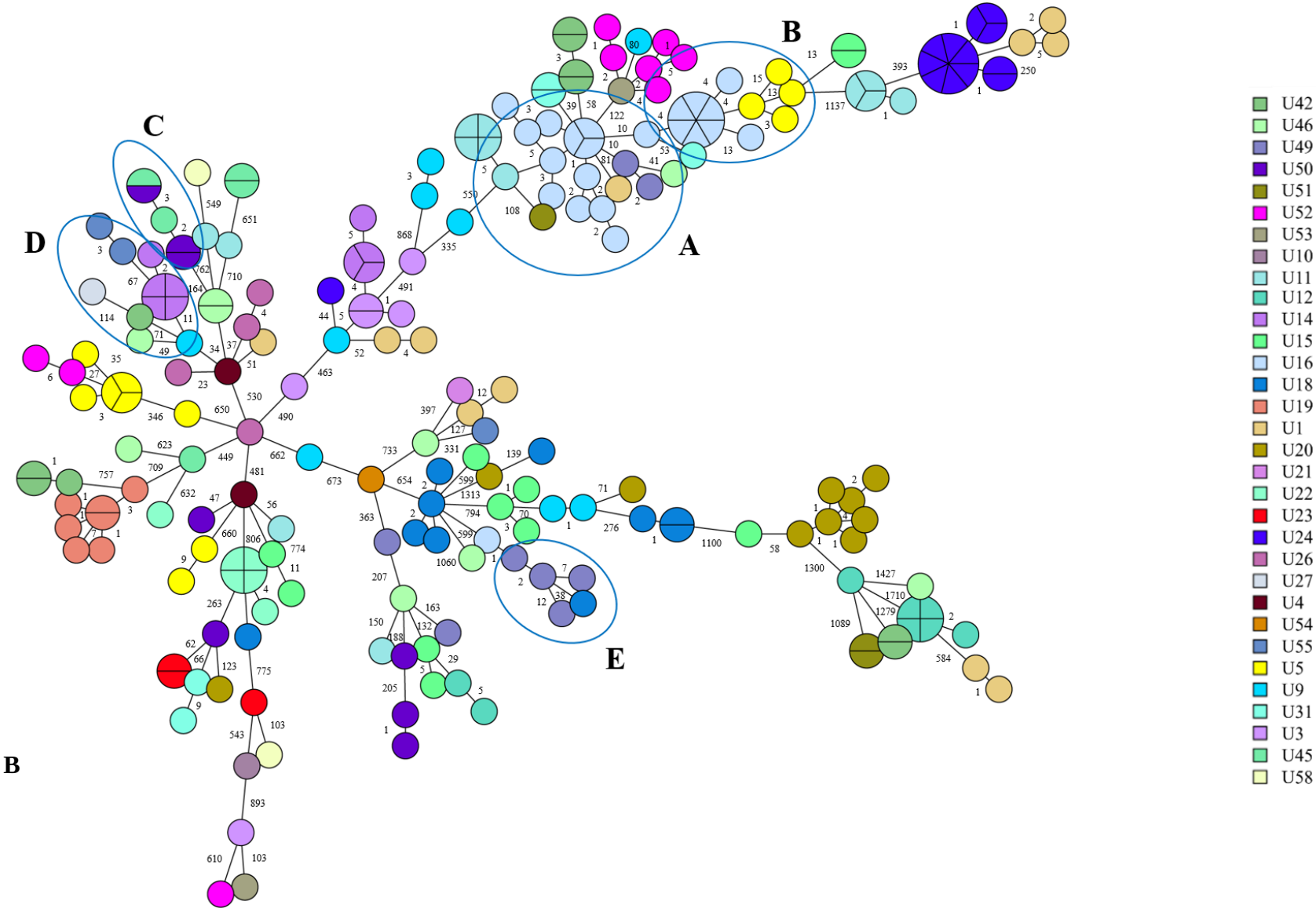
ST54
ST54
ST567
ST915
ST208
ST173
ST297
ST20
ST765

Panel A


A
B
C
D
E
U42
U46
U49
U50
U51
U52
U53
U10
U11
U12
U14
U15
U16
U18
U19
U1
U20
U21
U22
U23
U24
U26
U27
U4
U54
U55
U5
U9
U31
U3
U45
U58

Panel B

Figure 5.4. Minimum spanning trees (MSTs) based on whole genome multilocus sequencing typing (wgMLST) analysis of 4877 target genes of 224 isolates of *S. epidermidis* isolates from multiple anatomical sites (53 oral, 51 nasal, 55 periodontal pocket, 16 finger, 17 toe, 32 ulcer) of 33 distinct participants with DFUs investigated by wgMLST. Each node (or portion of a node) represents one distinct isolate. The numbers aligned with each branch indicate the numbers of allelic differences between neighbouring isolates. Isolates that differ from each other by ≤24 allelic differences are considered closely related based on previously described relatedness thresholds (129, 315) and are indicated by their enclosure within large blue circles. **Panel A:** MST indicating distinct sequence types (STs) according to colour, identified amongst isolates from multiple anatomical sites of 32 distinct participants. The coloured legend indicates STs identified in the isolate population. **Panel B:** MST showing isolates recovered from distinct participants illustrated by corresponding colours as indicated in the legend along with patient numbers. Abbreviations: U; Ulcer, ST; Sequence type.

5.3.2.3. Genes encoding antibiotic resistance in S. epidermidis *isolates from patients without and with DFUs.*

The prevalalence of genes encoding antibiotic resistance was investigated amongst the *S. epidermidis* isolates subjected to wgMLST from patients with and without DFUs. Overall, 11 different genes were detected (*blaZ*, *mph*(C), *msr*(A), *fusB*, *fusC*, *tetK*, *dfrS1*, *erm*(C), *aacA-aphD*, *cat*, and *qacA*).

The most commonly found resistance gene identified among isolates from both populations was the beta lactam resistance gene *blaZ* (Table 5.5). The *blaZ* gene was significantly more prevalent (P<0.05) in isolates recovered from patients without DFUs (46/103 [44.6%] from nine participants) than patients with DFUs (74/224 [33.0%] from 12 participants). The fusidic acid-encoding resistance gene *fusB* was also identified from isolates recovered from both patient cohorts. This gene was significantly more prevalent (P<0.01) in patients without DFUs (40/103 [38.8%] from seven participants) than patients with DFUs (26/224 [11.6%] from three participants) (Table 5.5). The macrolide resistance genes *erm*(C), *mph*(C) and *msr*(A) were detected in both populations and were highly prevalent in isolates recovered from patients with and without DFUs. The *erm*(C) gene was identified in isolates recovered from both patients with (10/224 [4.4%] from four participants) and without DFUs (12/103 [11.6%] from four participants). It was more prevalent in isolates recovered from patients without DFUs (11.8%). The *mph*(C) gene was significantly more prevalent (P<0.01) in isolates recovered from patients without DFUs (60/103 [58.2%] from seven participants) than with DFUs (15/224 [6.6%] from three participants) (Table 5.5). Also, *msr*(A) was also significantly more prevalent (P<0.01) in isolates recovered from participants without DFUs (41/103 [39.8%] from six participants), than participants with DFUs (12/224 [5.3%] from three participants),

Other genes identified less frequently across the isolate population included *qac*(A) (21/103 [20.7%] from seven participants without DFUs*;* 15/224 [6.6%] in two patients with DFUs), *tetK* (18/103 [17.8%] in three patients without DFUs; 6/224 [2.6%] in two patients with DFUs), *dfrS1* (1/103 [0.9%] in one patient without DFUs; 7/224 [3.1%] in two patients with DFUs).

Resistance genes only identified within isolates recovered from patients with DFUs include *merA* (1/224 [0.4%] in one patient with a DFU), *merB* (1/224 [0.4%] in one patient with a DFU), and *aacA-aphD* (3/224 [1.3%] in one patient with a DFU) (Table 5.5).

Table 5.5. Antimicrobial agent resistance and virulence genes detected in *S. epidermidis* isolates from patients with and without DFUs

Sample Site	Participants (*N*)	Resistance Genes	Virulence Genes (including ACME[a])	SCC/SCC*mec* type/genes detected	Presumptive SCC*mec* type
Patients without DFUs					
OR	7	*blaZ* (17), *erm*(C) (4), *fusB* (18), *mph*(C) (18), *msr*(A) (11), *qacA* (4), *tetK* (4), dfrS1 (1), *mecA* (2)	I (3), II (13), III (26), IV (4)	NA	NA
N	10	*blaZ* (31), *erm*(C) (4), *fusB* (2), *fusC* (1), *mph*(C) (28), msr(A) (18), *mph*(C) (3), *qacA* (11), *tetK* (5), *mecA* (3)	*hlg*(A) (2), *luk*(X/Y) (2), I (5), II (18), III (18), IV (10), V (1), VI (1)	*ccrAB4* (1), *qgpA* (2), *mecR1* (2)	IV (1)
PP	4	*blaZ* (16), *erm*(C) (2), *fusB* (15), *mph*(C) (8), *msr*(A) (9), *qacA* (2), *tetK* (7), *mecA* (3)	II (6), III (14), IV (3)	*ccrAB2* (2), *qgpA* (2), *mecR1* (2)	IVc (2)
F	3	*blaZ* (6), *erm*(C) (2), *fusB* (5), *mph*(C) (2), *msr*(A) (3), *qacA* (3), *tetK* (2), *mecA* (3)	I (2), II (1), III (3)	*ccrAB2* (2), *qgpA* (2), *mecR1* (2)	IVc (2)
T	2	*blaZ* (4), *fusC* (4), *qacA* (1), *mph*(C) (1), *mecA* (4)	II (5)	NA[b]	NA
Patients with DFUs					
OR	4	*blaZ* (11), *fusB* (8), *fusC* (2), *mph*(C) (7), *msr*(A) (3), *qacA* (6), *tetK* (1), *dfrS1* (1), *mecA* (4)	*sec* (1), *hlg*(A) (1), *luk*(X/Y) (1), *ssp*(A) (1), I (4), II (21), III (3), IV, (4), V (3), VI (1)	*ccrAB2* (3), *qgpA* (3), *mecR1* (3)	IVc (3)
N	2	*blaZ* (6), *msr*(A) (2), *erm*(C) (3), *fusB* (3), *mph*(C) (2), *aacA-aphD* (2), *qacA* (4),	I (10), II (6), III (11), IV (2), V (2)	*ccrAB2* (1), *qgpA* (1), *mecR1* (1)	IVc (1)
PP	4	*blaZ* (12), *erm*(C) (1), *fusB* (9), *mph*(C) (4), *msr*(A) (6), *qacA* (1), mecA (3), *tetK* (3)	I (15), II (18), III (3), IV (2), V (2)	*ccrAA* (1), *ccrAB2* (3), *qgpA* (3), *mecR1*	IVc (3)
F	3	*blaZ* (9), *mph*(C) (1), msr(A) (1), *fusB* (3), *fusC* (1), *tetK* (1), *tetK* (4), *dfrS1* (5), *erm*(C) (4), *aacA-aphD* (1), *cat* (1), *qacA* (3)	I (4), II (6), III (3), IV (2), V (2)	*ccrAB2* (2), *qgpA* (2), *mecR1* (2)	IVc (2)
T	2	*erm*(C) (1), *mecA* (1)	I (2), II (7), III (10), IV (1), VI (2)	*ccrAB2* (1), *qgpA* (1), *mecR1* (1)	IVc (1)
U	4	*blaZ* (8), *merA* (1), *merB* (1), *fusB* (3), *dfrS1* (1), *qacA* (1), *erm*(C) (1), *mecA* (2)	I (1), II (13), IV (2), V (2),	NA	NA

[a] Numbers I-VI denote ACME types in this column only.

[b] No SCC*mec* elements were identified in isolates recovered from this sample site.

Abbreviations: OR; Oral Rinse, N; Nares, PP; Periodontal Pocket, F; Finger, T; Toe, U; Ulcer, NA; Not applicable.

The *mecA* gene was identified in both population groups (*N*=23) and was more prevalent in isolates recovered from patients without DFUs (15/103 [14.6%] from five distinct patients) than in isolates recovered from patients with DFUs (9/224 [4.0%] from three distinct patients) (Table 5.5).

*5.3.2.4. Genes encoding SCC*mec *types in* S. epidermidis *isolates from patients without and with DFUs.*

Overall, 15 isolates of *S. epidermidis* harboured SCC*mec* IV as previously described (71). The predominant SCC*mec*-associated genes detected included the class B *mec* (*mecA, mecR1,* ugpQ) genes and *ccrAB2*, indicative of SCC*mec* IVc, from both patients with and without DFUs (Table 5.5). The predominant *ccr* genes identified were *ccrAB2* (*N*=14), identified in three patients with DFUs (*N*=10), and were identified in one patient without a DFU. The *ccrAB4* gene (*N*=1) was identified in the nares of the same distinct patient without a DFU, along with class B *mec*, indicative of SCC*mec* class IV. The SCC*mec* open reading frame associated *ccrAA* gene was identified in a distinct patient with a DFU (Table 5.5).

5.3.2.5. Genes encoding virulence factors in S. epidermidis *isolates from patients without and with DFUs.*

Virulence factors were identified in isolates of *S. epidermidis* investigated by wgMLST from multiple anatomical sites of patients both without and with DFUs (Table 5.5). However, with the inclusion of ACME types, five virulence factor encoding determinants were identified in the entire cohort of patients (*sec*, *hlgA, lukX/Y*, *ssp*(A), ACME). Furthermore, eight of the previously noted genes were detected in isolates from both patient cohorts (*hlgA, lukX/Y*, ACME) (Table 5.5).

The γ-haemolysin gene *hlgA* was detected in low prevalence within this population of *S. epidermidis* in contrast to investigation of virulence genes in *S. aureus* (Table 5.4). It was more prevalent in isolates from non-DFU patients (2/103 [1.9%] from one participant) than in participants with DFUs (1/224 [0.4%] from one participant). The leukocidin encoding genes *lukX/Y* was identified in patients without (2/103 [1.9%] from one participant) and with DFUs (1/224 [0.4] from one participant).

The ACME genes were identified in isolates from all anatomical sites in both patient cohorts (Table 5.5). Type I ACME (harbouring the *arc* and *opp3* operons) was

more prevalent in isolates recovered from people with DFUs (*N*=35 from 10 participants) than people without DFUs (*N*=10 from seven participants). Type II ACME (harbouring the *arcA* operon only) was more prevalent in isolates recovered from patients with DFUs (*N*=71 from 15 patients) than patients without DFUs (*N*=43 from 11 patients). Type III ACME (harbouring the *opp3* operon only) was the most prevalent ACME type identified in the *S. epidermidis* population (*N*=91) and was more prevalent in isolates recovered from patients without DFUs (*N*=61, from 10 individual patients) than from patients with DFUs (*N*=30 from nine individual patients). Type IV ACME (harbouring the *arcA* and *kdp* operons) was more prevalent in *S. epidermidis* isolates recovered from patients without DFUs (*N*=21) than in patients with DFUs (*N*=9). Type V ACME (harbouring *the arcA, opp3* and *kdp* operons) was more prevalent in isolates recovered from patients with DFUs (*N*=11, from seven patients) than in patients without DFUs (*N*=1, from one patient). This ACME type was most prevalent in *S. epidermidis* isolates recovered from the oral cavity, detected in three patients with DFUs (*N*=10). Type VI ACME (harbouring the *kdp* operon only) was more prevalent in isolates recovered from patients with DFUs (*N*=3) than in patients without DFUs (*N*=1). In the present study, ACMEs harbouring the *kdp* operon were detected in isolates recovered predominantly from the nares (*N*=13), oral cavity (*N*=8) and periodontal pockets (*N*=7) from both participant groups.

5.3.3. Summary of *S. epidermidis* and *S. aureus* populations between DFU and Non-DFU participants

Isolates from patients with DFUs harboured 16 distinct STs within the population, while isolates from patients without DFUs harboured 25 distinct STs within the population (Figs. 5.1 and 5.2). The overall population of *S. aureus* between both patient cohorts was composed of fewer distinct STs (*N*=20) in contrast to *S. epidermidis* between both patient groups (*N*=51) (Table 5.6).

The comparison of *S. aureus* and *S. epidermidis* populations amongst people with diabetes both with and without DFUs revealed that the *S. epidermidis* populations were more diverse than *S. aureus* populations, both in patients with and without DFUs. In total, 16 distinct *S. aureus* STs were identified among 266 isolates recovered from 42 participants with DFUs (16/42 [38.1%]), in contrast to the identification of 15 distinct STs from 105 isolates recovered from 28 participants (15/28 [53.6%]) without DFUs.

Table 5.6. Comparison of sequence types identified for *S. aureus* and *S. epidermidis* isolates from participants with and without DFUs

Species	Patient group (DFU/NU)	No. of distinct STs (Total)	No. of isolates WGS (%) [a]	No. of participants (%)
S. aureus	DFU	16	266 (38.1%)	42 (33.9%)
	Non-DFU	15	105 (15.1%)	28 (22.5%)
	Total	20	371 (53.1%)	70 (56.4%)
S. epidermidis	DFU	48	224 (32.1%)	36 (29.1%)
	Non-DFU	28	103 (14.7%)	18 (14.5%)
	Total	52	327 (46.9%)	54 (43.6%)

[a]The percentage totals are based on the total number of isolates investigated by whole genome sequencing.

In contrast, the numbers of distinct *S. epidermidis* STs detected was larger than the number of participants investigated for both patient cohorts. In total 48 distinct *S. epidermidis* STs were identified in 224 isolates recovered from 36 participants with DFUs, in contrast to the identification of 28 distinct STs from 103 isolates recovered from 18 participants without DFUs (Figs. 5.3 and 5.4).

Genes encoding both antibiotic resistance and virulence genes in *S. aureus* were more prevalent across isolates from patients with DFUs in comparison to patients without DFUs (Table 5.3). The highest prevalence of virulence (N=109) and antibiotic resistance (N=142) genes were identified from *S. aureus* isolates recovered from ulcers of patients with DFUs (Table 5.3).

The prevalence of antibiotic resistance-encoding genes within the populations of *S. epidermidis* was similar in both patient groups. The *blaZ* gene was the most frequently identified antimicrobial resistance gene identified in isolates from the entire population of participants, both without and with DFUs (Table 5.5). Virulence factor-encoding genes were more prevalent in isolates recovered from participants with DFUs, including ACME types (Table 5.5). ACME type II was the most prevalent ACME type among isolates from both patient populations (Table 5.5).

5.3.3. Comparison of *S. aureus and S. epidermidis* isolates recovered from multiple anatomical sites of the same individual by wgMLST.

Of the 37 patients from whom isolates of either *S. aureus* or *S. epidermidis* were identified from oro-nasal and ulcer sites, 23/36 (63.9%) harboured closely-related isolates from both the oral cavity and the DFU. This finding, that 23 patients harboured highly-related isolates, and only 13 did not, was significant ($P<0.05$).

Comparative WGS was performed on multiple *S. aureus* isolates from distinct anatomical sites from 26 patients with ulcers (patients U3, U7, U12, U14, U15, U18, U21, U25, U26, U33, U34, U40, U41, U43, U44, U47, U48, U51, U55, U56, U57, U58, U61, U63, U67, and U74) from whom *S. aureus* was recovered from both oro-nasal and ulcer sites (Table 5.7, Fig. 5.5). Of these 26 patients, 19 (73.1%%) patients yielded isolates from oro-nasal and ulcer sites that were genetically indistinguishable. Of these 19 patients with closely related oro-nasal and DFU isolates, 5/19 (3.6%) had closely related isolates from the finger, and 5/14 (3.6%) had closely related isolates from the toe. Of the 19 patients with closely related oro-nasal and DFU isolates, the nares (14/19 [73.6%%]) most commonly yielded isolates with the same ST as isolates from DFUs.

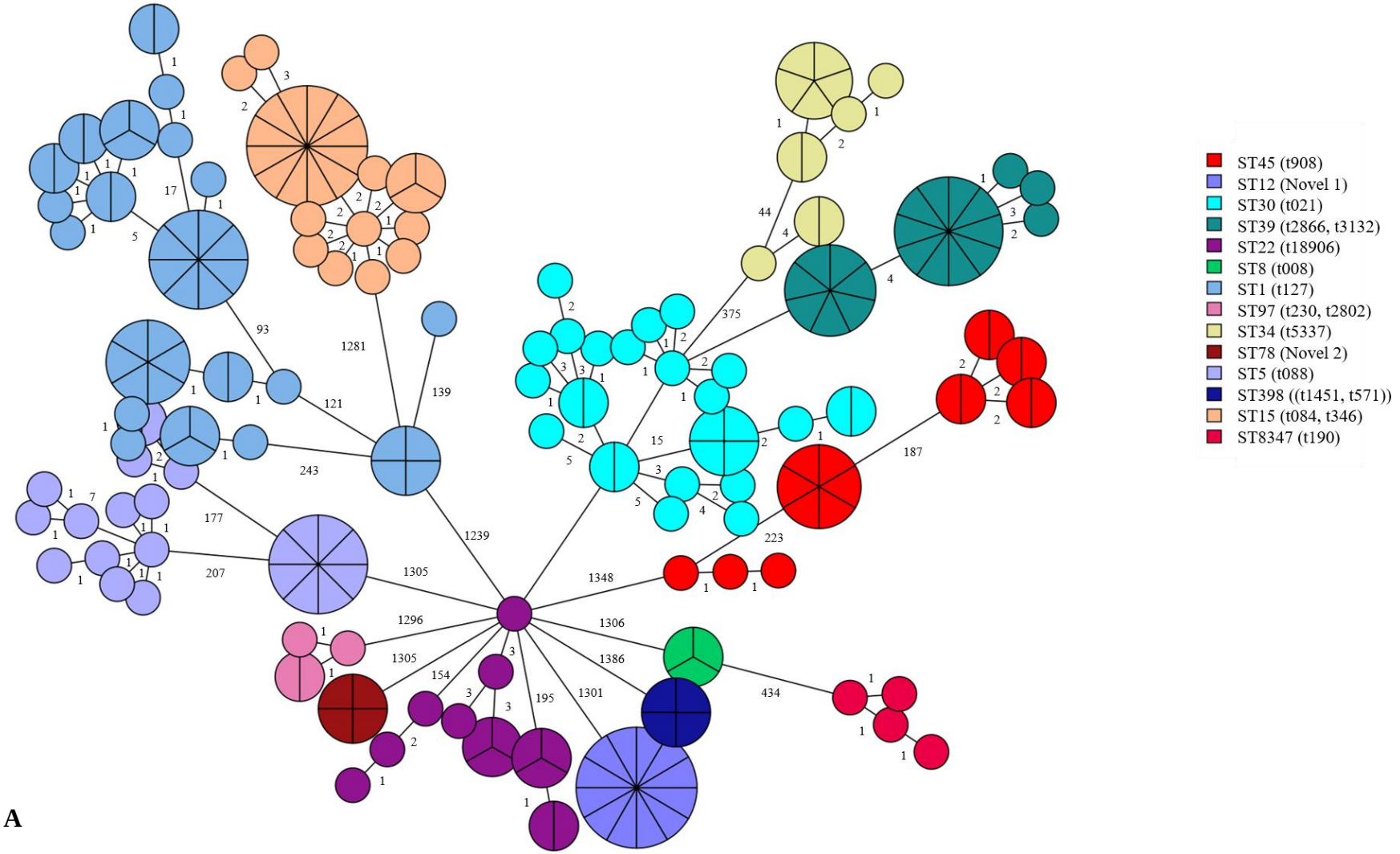
Comparative WGS was performed on multiple *S. epidermidis* isolates from distinct anatomical sites from 10 patients with DFUs (U1, U5, U11, U16, U24, U42, U45, U46, U49 and U50) (Table 5.8, Fig. 5.6) from whom *S. epidermidis* was recovered from both oro-nasal and ulcer sites. Identical STs were identified in oro-nasal and ulcer sites in 4/10 (40%) of participants investigated (Table 5.8, Fig. 5.6). Of these four patients with closely related oro-nasal and DFU isolates, one had a closely related isolate from the finger. Of the four patients, isolates from periodontal pockets (3/5 [60%]) most commonly had the same ST as the ulcer isolate.

Table 5.7. Sequence types (STs) and *spa* types identified amongst *S. aureus* isolates from multiple distinct anatomical sites of each *S. aureus*-positive participant with DFUs.

Patient	Oro-Nasal Sites (*N*)			Finger (*N*)	Toe (*N*)	Ulcer (*N*)	ST (average and range of allelic differences)
	Nasal Swabs (*N*)	Oral Cavity (*N*)	Periodontal Pockets (*N*)				
U3	ST97 (2)	ST45 (2)	NR	NR	NR	ST97 (2)	ST97 (0.5, 0-1), ST45 (0)
U7	NR	ST45 (4)	NR	NR	NR	ST1 (6)	ST45 (0.25, 0-1), ST1 (1, 2)
U12	ST39 (8)	ST39 (3)	ST39 (2)	NR	NR	ST39 (7)	ST39 (0.5, 0-4)
U14	ST30 (6)	NR	NR	NR	ST30 (4)	ST30 (7)	ST30 (0.25, 0-11)
U15	NR	ST39 (1)	NR	NR	NR	ST22 (3)	ST22 (0, 0), ST39 (0)
U18	NR	NR	ST22 (2)	NR	NR	ST22 (1)	ST22 (2, 0-3)
U21	ST34 (5)	ST34 (5)	ST1 (1)	NR	ST1 (4)	ST1 (5)	ST34 (0.11, 0-1), ST1 (0, 0)
U25	ST34 (3)	NR	NR	NR	NR	ST8 (3)	ST34 (0.3, 0-1), ST8 (0, 0)
U26	ST30 (3)	ST30 (3)	NR	NR	NR	ST45 (3)	ST30 (0.28, 3-7), ST45 (1, 0-1)
U33	NR	NR	ST15 (4)	NR	NR	ST398 (3)	ST15 (0.5, 0-1) ST398 (0.5, 0-1)
U34	ST30 (4)	ST30 (3)	NR	NR	NR	ST78 (3)	ST30 (1.42, 1-2), ST78 (1.25, 0-4)
U40	ST15 (3)	ST15 (3)	NR	ST15 (2)	NR	ST15 (4)	ST15 (0.67, 0-2)
U41	ST22 (1)	ST22 (1)	NR	ST22 (1)	ST22 (1)	ST22 (1)	ST22 (0.4, 0-2)
U43	ST1 (2)	NR	NR	ST1 (1)	NR	ST1 (1)	ST1 (0, 0)
U44	ST45 (1)	ST45 (2)	NR	NR	ST45 (1)	ST45 (1), ST1 (1)	ST1 (0, 0-0), ST45 (0.83, 2)
U47	ST5 (3)	ST5 (3)	ST5 (1)	NR	ST5 (1)	ST5 (2)	ST5 (0.38, 1-10)

U48	ST22 (1)	NR	NR	NR	ST22 (1)	ST22 (1)	ST22 (1, 1)
U51	ST1 (1)	NR	NR	NR	NR	ST1 (1)	ST1 (2, 4)
U55	ST15 (3)	ST15 (3)	NR	NR	NR	ST15 (3)	ST15 (1.6, 1-3)
U56	NR	ST8347 (1)	NR	NR	NR	ST8347 (3)	ST8347 (0, 0)
U57	ST1 (3)	ST1 (2)	NR	NR	NR	ST1 (3)	ST1 (2.12, 1-3)
U58	ST5 (4)	NR	NR	NR	NR	ST5 (4)	ST5 (0.125, 0-1)
U61	ST1 (3)	NR	NR	ST1 (1)	NR	ST1 (3)	ST1 (5.14, 1-27)
U63	ST12 (3)	ST12 (3)	NR	ST12 (3)	NR	ST12 (3)	ST12 (0.33, 0-1)
U67	ST1 (2)	ST1 (3)	NR	NR	ST1 (2)	ST1 (2)	ST1 (0.88, 0-2)
U74	NR	ST5 (2)	NR	NR	NR	ST5 (2)	ST5 (1, 1-2)

Abbrevation: NR; Not recovered, U; Ulcer, ST; Sequence type.


ST45 (t908)
ST12 (Novel 1)
ST30 (t021)
ST39 (t2866, t3132)
ST22 (t18906)
ST8 (t008)
ST1 (t127)
ST97 (t230, t2802)
ST34 (t5337)
ST78 (Novel 2)
ST5 (t088)
ST398 ((t1451, t571))
ST15 (t084, t346)
ST8347 (t190)
93
1281
139
121
243
1239
177
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1296
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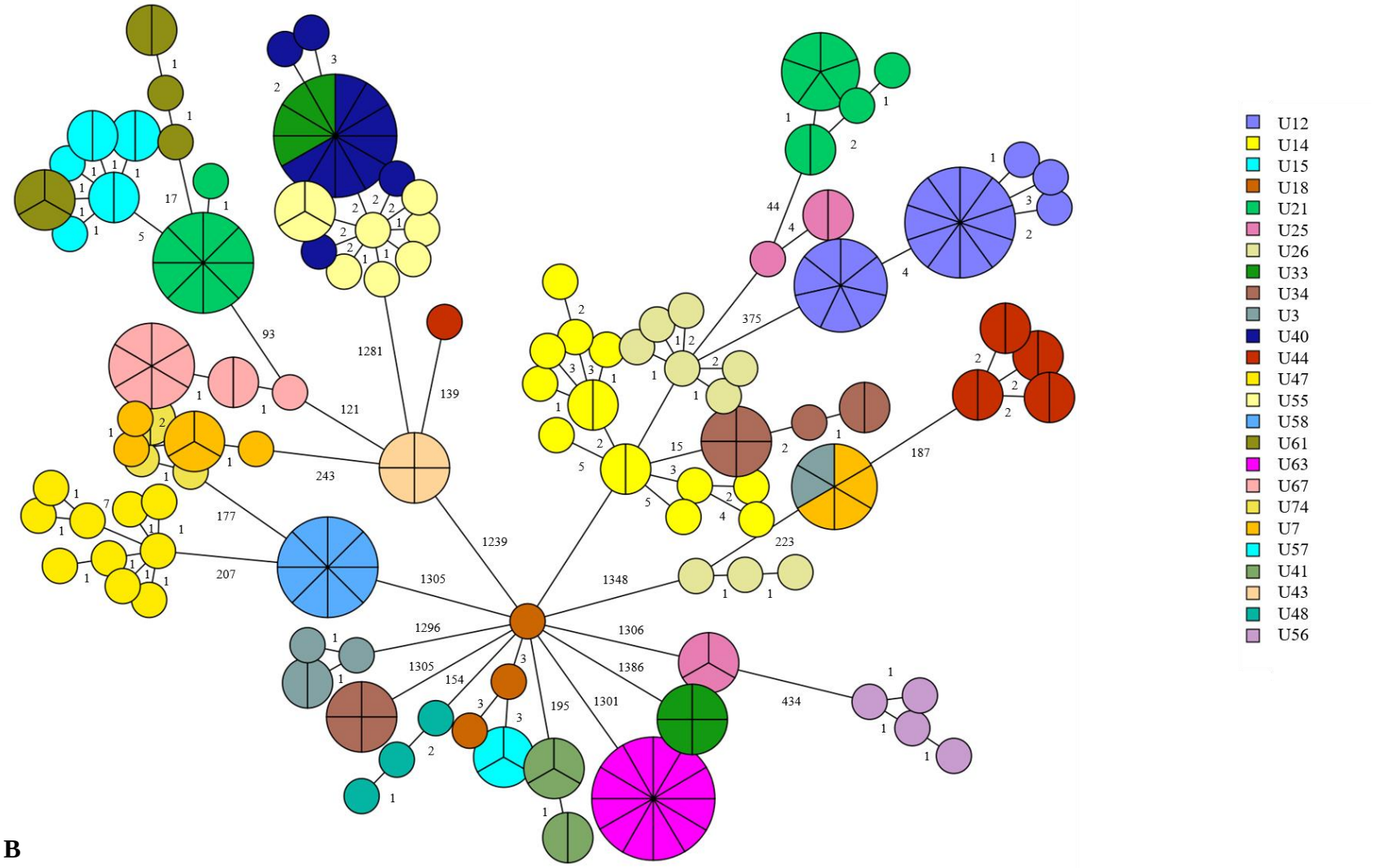
1305
1386
154
195
1301
434
375
44
187
223
15

Panel A


U12
U14
U15
U18
U21
U25
U26
U33
U34
U3
U40
U44
U47
U55
U58
U61
U63
U67
U74
U7
U57
U41
U43
U48
U56

Panel B

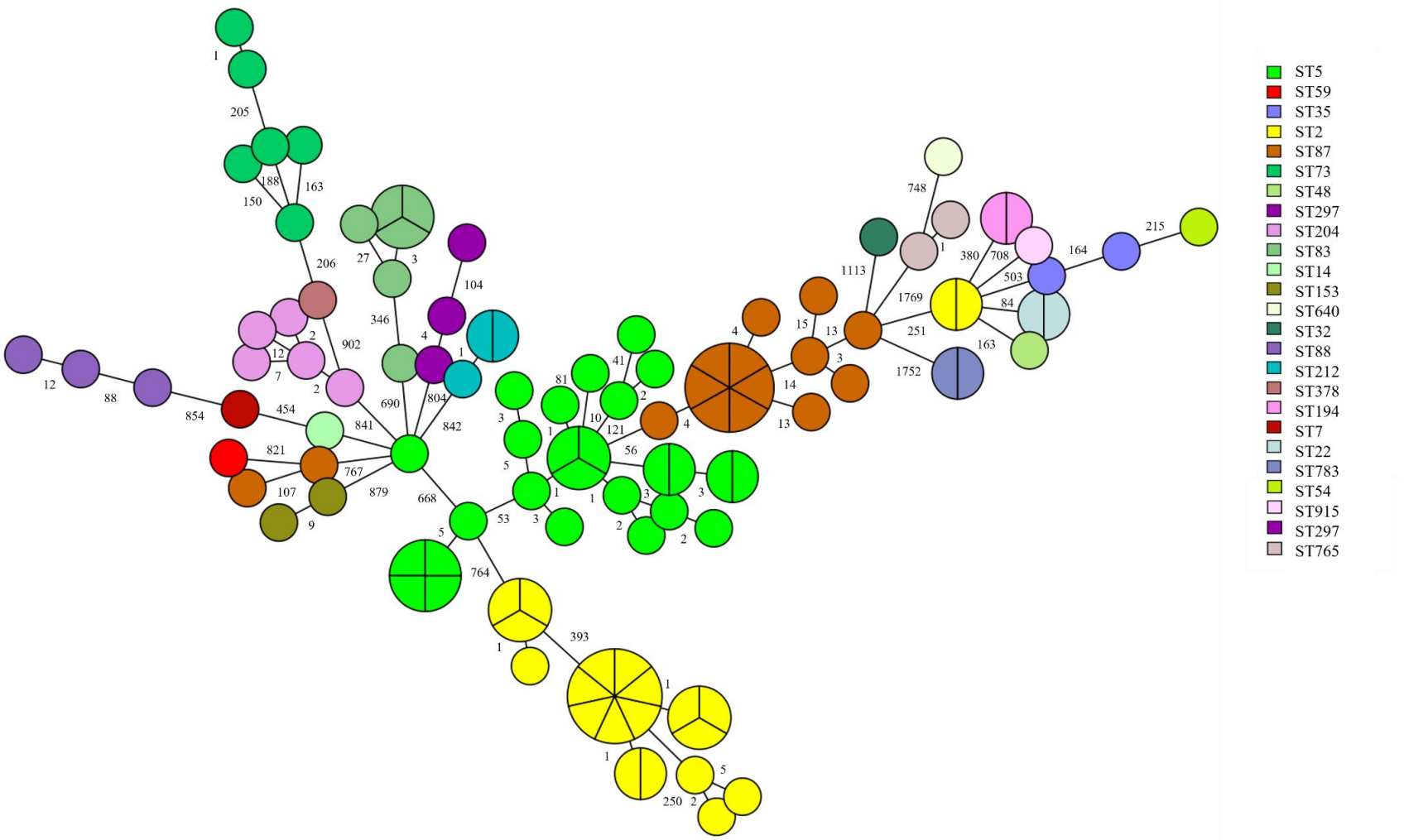
Figure 5.5. Minimum spanning trees (MSTs) based on whole genome multilocus sequence typing (wgMLST) analysis 3,904 target genes for 215 isolates of *S. aureus* from multiple anatomical sites (44 oral, 56 nasal, 10 periodontal pocket, 6 finger, 18 toe and 81 ulcer) of 26 distinct participants with DFUs investigated by whole genome multilocus sequence typing (wgMLST). Each node (or portion of a node) represents one distinct isolate. The numbers aligned with each branch indicate the numbers of allelic differences between neighbouring isolates. Isolates that differ from each other by ≤24 allelic differences are considered closely related as previously described (87, 309, 310). **Panel A:** Minimum spanning tree indicating distinct sequence types (STs) according to colour, identified amongst isolates from multiple anatomical sites of 26 distinct participants. The coloured legend indicates STs and corresponding *spa* types associated with each ST, where applicable. **Panel B:** Minimum spanning tree constructed as per the MST indicated in Panel A, with isolates recovered from distinct participants illustrated by corresponding colours as indicated in the legend along with patient numbers. Abbreviations U; Ulcer, ST; Sequence Type.

Table 5.8. Sequence types of isolates of *S. epidermidis* recovered from multiple anatomical sites from patients with DFUs

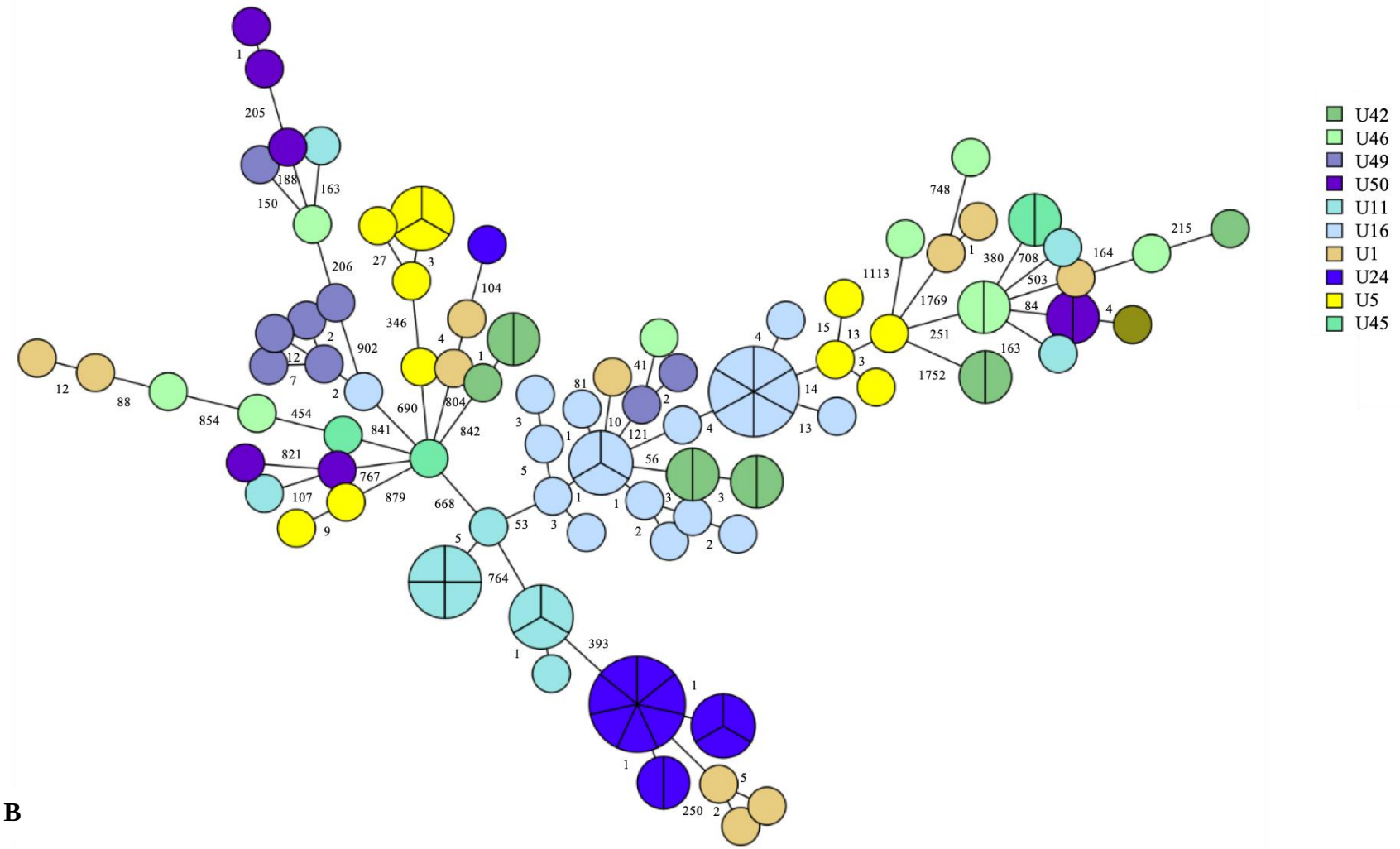
Patient	Oro-nasal Sites (*N*)			Finger (*N*)	Toe (*N*)	Ulcer (*N*)	ST (average and range of allelic differences)
	Nasal Swabs (*N*)	Oral Cavity (*N*)	Periodontal Pockets (*N*)				
U1	ST88 (1)	ST35 (1) ST88 (1)	ST5 (1) ST2 (3)	NR	ST765 (2)	ST297 (2)	ST88 (6, 12), ST35 (0, 0), ST1 (0, 0), ST2 (2.33, 2-5), ST765 (0.5, 1), ST297 (2, 4)
U5	ST83 (3) ST87 (1)	ST83 (1)	ST83 (1) ST87 (2)	ST153 (2)	ST87 (1)	ST83 (1)	ST83 (62.6, 0-346), ST87 (8.25, 3-15), ST153 (4.5, 9)
U11	NR	ST87 (1)	ST48 (1) ST723 (1) ST915 (1)	NR	NR	ST2 (4) ST5 (5)	ST2 (0.25, 0-1), ST48 (0, 0), ST5 (1, 0-1), ST723 (0, 0), ST87 (0, 0), ST915 (0, 0)
U16	ST5 (1) ST87 (3)	ST5 (2)	ST5 (7)	ST5 (2) ST204 (1)	NR	ST87 (6)	ST204 (0, 0), ST5 (1.74, 0-5), ST87 (2.33, 0-13)
U24	ST2 (3) ST297 (1)	ST2 (2)	ST2 (3)	NR	NR	ST2 (4)	ST2 (0.16, 0-1), ST297 (0, 0)
U42	ST5 (2)	ST278 (1)	NR	NR	NR	ST212 (2)	ST212 (0, 0), ST278 (0, 0), ST5 (0.5, 0-1)
U45	NR	ST22 (1)	NR	ST22 (1)	NR	ST14 (1)	ST22 (1.5, 3), ST14 (0, 0)
U46	NR	ST35 (1)	ST5 (1) ST32 (1)		NR	ST640 (1)	ST32 (0, 0), ST35 (0, 0), ST5 (0, 0), ST640 (0, 0)

U49	NR	ST204 (2)	ST204 (1) ST5 (2) ST73 (1)	ST38 (1)	NR	ST204 (1)	ST204 (5.25, 2-12), ST38 (0, 0), ST5 (1, 2), ST73 (0, 0)
U50	ST87 (1)	NR	ST73 (1)	NR	NR	ST73 (2)	ST73 (68.6, 1-205), ST87 (0, 0)

Abbreviation: NR – None recovered


ST5
ST59
ST35
ST2
ST87
ST73
ST48
ST297
ST204
ST83
ST14
ST153
ST640
ST32
ST88
ST212
ST378
ST194
ST7
ST22
ST783
ST54
ST915
ST297
ST765

Panel A



Panel B

Figure 5.6. Minimum spanning trees (MSTs) based on whole genome multilocus sequence typing (wgMLST) analysis of 4877 target genes for 111 isolates of *S. epidermidis* from multiple anatomical sites (17 oral, 22 nasal, 35 periodontal pocket, 6 finger, 5 toe and 26 ulcer) of 10 distinct participants with DFUs. Each node (or portion of a node) represents one distinct isolate. The numbers aligned with each branch indicate the numbers of allelic differences between neighbouring isolates. Isolates that differ from each other by ≤24 allelic differences are considered closely related as previously described (87, 315). **Panel A:** Minimum spanning tree indicating distinct sequence types (STs) according to colour, identified amongst isolates from multiple anatomical sites of 11 distinct participants. The coloured legend indicates STs associated with each ST, where applicable. **Panel B:** Minimum spanning tree constructed as per the MST indicated in Panel A, with isolates recovered from distinct participants illustrated by corresponding colours as indicated in the legend along with patient numbers. Abbreviations: U (Ulcer); ST (Sequence Type).

5.4. Discussion

The previously published evidence suggesting that the anterior nares are an endogenous reservoir for *S. aureus* based DFUIs is of important clinical significance (98, 99, 255, 316). People who have diabetes are more prone to developing PD (316) and previous research from this department reported that PD results in significantly increased prevalence and abundance of staphylococci in the oral cavity, particularly *S. aureus* and *S. epidermidis* (48). To date, there has been no large scale comprehensive investigaton into the oral cavity and periodontal pockets as an endogenous reservoir for DFUIs in people with type II diabetes. McManus *et al.* (2020) previously implicated the oro-nasal cavity as a likely endogenous reservoir for DFUIs using wgMLST in a small-scale pilot study which provided valuable evidence that the oro-nasal cavity is a potential endogenous source of DFUI infection (99). The present study carried out a similar, more detailed investigation on a larger scale to provide further, detailed information on the oral health status and staphylococcal populations amongst people with diabetes in Ireland and the role of oro-nasal staphylococci in DFUIs.

5.4.1. *Staphylococcus aureus*

Staphylococcus aureus isolates from patients without DFUs investigated by WGS harboured ST45, ST12, ST30, ST39 and ST5. The most predominantly identified ST was ST45 (Fig. 5.1, Panel A). The predominant STs identified among DFU patients were ST12, ST15, ST30; ST39 and ST5 (Fig. 5.2), which have been commonly reported amongst European strains of MSSA (280).

Unsurprisingly, genes encoding antibiotic resistance were more prevalent in participants with DFUs, likely as a result of previous recurrent antibiotic treatments (Table 5.3). Surprisingly, the *mecA* gene was detected in low prevalence and was the least common resistance gene identified amongst *S. aureus* isolates recovered. In contrast, other studies previously reported a high prevalence of MRSA in DFUs (317-319). In the present study, all of the study participants were outpatients and it is possible that this reduced hospital exposure may have contributed to the reduction in MRSA prevalence in this cohort.

Resistance to penicillin in staphylococci is mediated by *blaZ*, the gene encoding beta-lactamase (320). The *blaZ* gene was the most prevalent gene identified in the current investigation and was significantly more prevalent in isolates recovered from patients with DFUs ($P<0.01$) than in isolates from patients without DFUs. These findings support the results of previous investigations involving the molecular characterisation of *S. aureus* from people with diabetes and DFUs that identified *blaZ* as the most common antibiotic resistance

gene in the study population (98, 99). The only other gene significantly more prevalent in participants with DFUs was *fosB* ($P<0.01$). A recent investigation suggested a possible transfer of *fosB* to staphylococci from *Enterococcus faecium* (321) indicating potential transfer of these genes within the healthcare environment.

Virulence factors were also more prevalent in isolates of *S. aureus* recovered from patients with DFUs than patients without DFUs (Table 5.3). The *lukD, hlIII* and *splB,* genes were significantly more prevalent ($P<0.01$, $P<0.05$ and $P<0.01$, respectively) in isolates investigated from participants with DFUs. The leukocidin encoding gene, *lukD,* has been significantly associated with infected ulcers as well as being used as marker for differentiation between infected and uninfected DFUs, and as a predictor for the outcome of grade 1 DFUs (322, 323). Similarly, *lukD/E* was highly prevalent in nasal and DFU sites reported by Dunyach-Remy (2017) as the most prevalent virulence factor identified (98). Although prevalent in the present study, *lukD/E* were not the most prevalent virulence genes (Table 5.3).

No PVL-positive *S. aureus* isolates were identified from participants without DFUs, while 35 isolates from seven patients with DFUs harboured PVL-positive strains. This toxin-encoding gene was more prevalent in the isolates from the nares of this patient cohort (12/188 [6.3%] from six participants). The high prevalence of PVL-harbouring strains in this anatomical site is likely due to the increased prevalence of *S. aureus* in this anatomical site in general, enabling the identification of more PVL-positive strains. The detection of PVL-positive *S. aureus* isolates within DFUs may suggest a poorer prognosis as PVL-positive isolates have been strongly associated with skin and soft tissue disease (324). Further screening of *S. aureus* isolates harboured by people with DFUs is therefore of great importance and could potentially be used as a clinical predictive marker for future severe *S. aureus*-based DFUIs.

5.4.2. *Staphylococcus epidermidis*

To date, there has been no comprehensive investigation of *S. epidermidis* populations in people with diabetes, although this organism has previously been recovered from DFUs utilising clinical swabs and bone biopsies (99, 291, 325).

This population diversity of *S. epidermidis* was apparent in the present study, when multiple isolates recovered from the same anatomical site of 14 participants without DFUs, and 27 patients with DFUs (Figs. 5.3 and 5.4) were identified as distinct STs. *Staphylococcus epidermidis* from DFUs and skin of people with DFUs has recently been investigated by WGS, identifying ST54, ST35 and ST640 as the predominant STs (291). There has been no

attempt to categorise oral *S. epidermidis* by ST to date, however previous studies have characterised oral and periodontal isolates of *S. epidermidis* by MLST, ACME and SCC*mec* typing (48, 302). Of the predominant STs (ST73, ST17, ST59, ST153 and ST329), ACME types (I, II and III) and SCC*mec* types (SCC*mec* IV and V) described by O'Connor *et al.* (2018) (48), only the ACME and *SCCmec* types correlated with the present investigation. However, although STs 73, 17, 59, 153 and 329 were identified in *S. epidermidis* in the present study, they were not the most prevalent (Table 5.4).

The increased prevalence and abundance of *S. epidermidis* in the oral cavity and periodontal pockets in people with PD and diabetes could be clinically significant, providing a microbial reservoir for DFUIs or a genetic reservoir for antimicrobial resistance genes for transfer to *S. aureus* DFUI-causing strains. Interestingly, ST73 was identified in 17/103 *S. epidermidis* isolates (16.5%), and from the oro-nasal cavity of 8/19 (42.1%) participants without DFUs (Fig. 5.3). It was also identified to a lesser extent in isolates recovered from patients with DFUs and was identified in 10/224 (4.5%) isolates from a further eight distinct participants (Fig. 5.4). From these isolates, 8/10 (80.0%) were identified in the oro-nasal cavity, and 2/10 (20.0%) from the DFU. Previous investigation of *S. epidermidis* populations in the oral cavities of people with PD also reported the recovery of three oral ST73 isolates, but no potential enrichment of this ST in this anatomical site was observed. (48, 302). In contrast to the general population diversity of *S. epidermidis* and common recovery of distinct STs from the same anatomical site on multiple occasions from both patient groups, the high prevalence of oro-nasal ST73 isolates in people with diabetes is suggestive of a possible enrichment of this lineage in this patient cohort. The identification of this ST in the oro-nasal cavity of both patient groups suggests that this enrichment may be independent of DFU status.

Previous research has suggested an enrichment of specific ACME types within distinct STs (302). However, this does not appear to be the case in the present study. Isolates identified as ST73 in both patient cohorts harboured ACME types II, III and IV. Similarly, ST5 harboured ACME types I-V and ST35 isolates harboured STs I-III, predominantly ACME type III (N=21). In general, ACME types were diverse amongst patient groups, sample sites, and ST lineages. It has long been understood that ACME improves fitness of *S. epidermidis* and enables it to colonise skin and mucosal membranes (53, 303). O'Connor *et al.* (2018) also reported the high prevalence of ACME-*arc* harbouring *S. epidermidis* isolates in periodontal pockets, and suggested it may be essential for the survival of *arc*-containing strains in oxygen deficient environments (48). With the exception of ACME VI, all ACME types were identified in isolates recovered from periodontal pockets. The ACME

types I and II, harbouring the *arc* gene, were the most prevalent in periodontal isolates from patients with DFUs, supporting the hypothesis previously suggested by O'Connor *et al.* (48, 302). However, in participants without DFUs, ACME type III, harbouring the *opp3* operon only, was three times more prevalent in periodontal isolates (Table 5.5).

Virulence factor and antimicrobial resistance encoding genes were identified in the population of *S. epidermidis,* although virulence factor genes were less prevalent amongst *S. epidermidis* isolates than the population of *S. aureus* (Tables 5.2 and 5.4). Similar to *S. aureus* populations, the most prevalent resistance gene identified from participants without and with DFUs was the *blaZ* gene. The *blaZ* gene has been reported as highly prevalent amongst CoNS populations (326) which is in agreement with the present study regarding *S. epidermidis*. The macrolide resistance encoding genes *mph*(C) *and msr*(A) were both significantly more prevalent in *S. epidermidis* isolates recovered from participants with DFUs than participants without DFUs ($P<0.01$). In contrast, *mph*(C) was only detected in isolates recovered from participants with DFUs, and *msr*(A) was more prevalent in the same patient population ($N=7$) than participants without DFUs ($N=1$), but not significantly more prevalent (Table 5.3). This data could reflect a common treatment pathway between strains, and also indicates which antibiotics could be avoided when identifiying treatment options for DFUIs, such as clindamycin, a macrolide antibiotic used to treat cross-resistance and emerging resistance in erythromycin-resistant *S. aureus,* used in the treatment of DFUIs (327).

There was a higher prevalence of SCC*mec*-associated genes from isolates recovered from participants with DFUs than patients without DFUs, potentially reflective of prior antibiotic exposure (Table 5.5). Similar to previous investigations involving SCC*mec* typing from *S. epidermidis* isolates, type II SCC*mec* and type II associated SCC*mec* elements were predominant (236, 328).

5.4.3. Identification of highly-related isolates from distinct patients

The presence of highly related *S. aureus* strains was detected in distinct patients on 28 occasions, including 15 and 13 patients with and without DFUs, respectively. On each occasion, closely related isolates recovered from distinct individuals belonged to the same ST and were separated by ≤ 15 allelic differences (Figs. 5.1 and 5.2, Panel B).

The presence of highly related *S. epidermidis* was also identified in distinct participants both and with ($N=9$) and without ($N=11$) DFUs. On each occasion, closely related isolates recovered from distinct individuals belonged to the same ST and were also

separated by ≤ 15 allelic differences (Figs. 5.3 and 5.4, Panels B) suggesting transmission of isolates between patients. The detection of closely related *S. epidermidis* isolates was particularly surprising due to the diverse population of this species observed in the present study both within individual participants as well as within specific anatomical sites.

In combination, the recovery of highly-related strains from distinct participants is suggestive of *S. aureus* and *S. epidermidis* transmission between participants, however, the participants involved did not always participate in the study on the same days. The number of participants on a given day varied, however the average sampling time between patients harbouring highly related isolates was 31.16 weeks (range two days-104 weeks).

Patients with DFUs visit the Diabetes Day Centre weekly, but participation in the present study only occurred on one occasion, therefore it is possible that patients with DFUs visited the clinic on the same days but did not necessarily participate in this study on the same day. However, the congregation of patients with open wounds in clinical waiting areas may may likely have aided this transmission to some degree and led to microbial transmission between some patients, either directly or indirectly via the environment. The possible release of fine particulate matter from the DFUs in clinical treatment areas due to debridement procedures, wound undressings and cleanings, may also have caused transmission of these microorganisms in the healthcare setting. This is less likely for patients without DFUs, who tend to visit the Diabetes Centre much less frequently, predominantly for an annual routine appointment with the Endocrinologist or diabetes nurse.

5.4.4. Oro-nasal and DFU isolate relatedness

Of 50 participants who yielded either *S. aureus* or *S. epidermidis* from the DFU, 36 (74.0%) also yielded the same species from the oral cavity as described in Chapter 4. Of these participants, the majority (23/36 [63.9%]), yielded highly related isolates (separated by ≤ 24 allelic differences (87, 309, 310)) from both oro-nasal and DFU sites. This provides convincing evidence for a route of transmission between these anatomical sites (Table 5.6, Figs. 5.5 and 5.6). The number of patients with highly-related oro-nasal and DFU isolates was significant ($P<0.05$), providing irrefutable novel evidence that individuals carrying oro-nasal *S. aureus* or *S. epidermidis* appear more likely to be susceptible to DFU colonisation by the same strain.

Highly related isolates of *S. aureus* were identified in 19/26 (73.1%) ONC and DFU sites in participants with DFUs (average 0.7, range 0-7 allelic differences) (Fig. 5.5).

It has been previously suggested that the nares are an endogenous reservoir for DFUIs (98). In the present investigtation, closely related or genetically indistinguishable

nasal and DFU isolates of *S. aureus* were identified in 15/26 (57.6%) participants, providing supporting evidence for a likely nasal *S. aureus* endogenous reservoir Interestingly, genetically indistinguishable oral and DFU isolates were identified in 13/26 (50.0%) of participants. Trafficking of *S. aureus* between oral and nasal cavities has been documented previously (99, 329) and the data from the present investigation suggests that the oral cavity could potentially play an important role as an endogenous *S. aureus* reservoir or as a route for systemic transmission.

Of the 10 ulcer patients from whom *S. epidermidis* was recovered from both oro-nasal and ulcer sites, the presence of highly related strains in both sites was detected in four patients (40.0%) (average 2.01, range 0-15 allelic differences) (Fig. 5.6) (99). Of these four patients, the periodontal pockets were the most common site yielding isolates that were genetically indistinguishable to the ulcer isolates, identified in three (75.0%) patients. Thus, it is possible that periodontal carriage of *S. epidermidis* is an endogenous risk factor for a *S. epidermidis*- based DFUI. Despite multiple isolates of *S. epidermidis* from the same anatomical sites often harbouring isolates with distinct STs, isolates recovered from the DFUs always harboured a consistently one ST per patient, suggesting the successful establishment of one lineage in the wound.

Many of the participants with DFUs investigated in the present study had shallow, superficial ulcers. Further investigations involving bone-deep DFUs where *S. epidermidis* has been reported in bone biopsies from DFU-related osteomyelitis (325) could be undertaken to identify the possible clinical significance of oral *S. epidermidis* in bone-deep DFUs. It would be necessary to investigate participants with deeper ulcers or osteomyelitis using the same techniques to identify how serious a risk oral carriage of *S. aureus* and *S. epidermidis* may be. It is also important to compare the prevalence and genetic relatedness of other organisms found at both sites, including comparative WGS analyses of anaerobic species from both deeper periodontal pockets and more advanced DFUs.

Of the 19 participants who yielded highly related *S. aureus* strains from oro-nasal and DFU sites, highly related *S. aureus* strains were also identified from either the finger or toe in 10 participants. A comparable finding was not observed for participants who yielded highly related *S. epidermidis* strains from oro-nasal and ulcer sites, although a larger scale investigation solely based on *S. epidermidis* may provide more information in this regard.

It is likely that skin contact and direct hand transfer plays an important role in transmission of these strains between these anatomical sites. However, this does not rule out possible bloodstream transmission via periodontal pockets, as periopathogens have been identified in the bloodstream after toothbrushing and other dental procedures, including

cleaning and extraction (233, 330, 331). Using WGS, it is practical to further investigate this possibility, particularly in patients with PD and oral *S. aureus* and *S. epidermidis.* Strains of *S. aureus* and *S. epidermidis* have also been recovered from the blood samples (332), with nasal carriage of *S. aureus* indicating a higher risk for bacteraemia (333), however no studies have been carried out identifying *S. aureus* and *S. epidermidis* in the blood of people with PD. Future investigations will be required to identify whether *S. aureus* or *S. epidermidis* bacteraemia originating from the oral cavity is a potential risk factor for DFUIs.

Highly related oro-nasal and DFU isolates of *S. aureus* and *S. epidermidis* were identified in 73.1% and 36.3% of participants, respectively, from whom these strains were recovered from both sites, which was significant as previously discussed ($P<0.05$). Thus, it is evident that the oral cavity and nares are likely reservoirs for staphylococcal-based DFUIs, consolidating the hypothesis proposed in the pilot investigation (99).

Chapter 6

General Discussion

There is an extremely high prevalence of diabetes worldwide, and global diabetes cases are projected to rise further from 539 million today to 1.3 billion by 2050 (334). In Western Europe, 89.3% of diabetes cases are classified as type II diabetes, and it is estimated that more than 200,000 people are living with diabetes in Ireland (334). Diabetic foot ulcers are a major complication and source of morbidity in people living with diabetes. The lifetime risk of developing a DFU is between 19-34% (335). People who develop a DFU have a lower extremity amputation risk of 20% and a five-year mortality rate of 50-70% following amputation (335). The incidence of DFUs in males is approximately 1.5 times higher than in females in patients with diabetes (25, 336). The risk of lower limb extremity amputation is significantly heightened by a DFUI (337), increasing morbidity and mortality, and reducing quality of life. *Staphylococcus aureus* is the leading cause of DFUIs, and research has identified the anterior nares, the primary ecological niche of *S. aureus* in humans, as an endogenous risk factor for *S. aureus* DFUIs (262).

Periodontal disease is so highly prevalent in people with diabetes that it is often considered another diabetes-related complication. Some studies have reported the prevalence of PD in people with diabetes to be as high as 90% (338, 339). Previous research from this laboratory reported that people with PD have an increased prevalence and abundance of staphylococci, particularly *S. aureus* and *S. epidermidis*, in the oral cavity and periodontal pockets (48). In consideration of these findings and the well-established bi-directional relationship between diabetes and PD (340-342), it was therefore important to investigate if people with diabetes harbour increased levels of staphylococci in the oral cavity. Furthermore, previous pilot research from this laboratory revealed the presence of identical strains of *S. aureus* at both the oro-nasal and ulcer sites of 6/13 patients with type II diabetes and DFUs (343) and highlighted an important requirement of further research in this area.

The present study is the first to comparatively investigate multiple staphylococcal species from distinct anatomical sites including the oro-nasal cavity and DFUs of people with type II diabetes using MALDI-TOF-MS, 16S rRNA sequencing and WGS technology. The use of chromogenic Sa*Select*™ medium for the recovery and presumptive identification of staphylococci enabled this research to directly compare and contrast multiple species of staphylococci between participants with type II diabetes with and without DFUs for the first time. Many previous investigations of this nature used various phenotypic or genotypic methods that were less discriminatory, failed to carry out thorough genotyping of collected isolates, or did not investigate isolates from multiple anatomical sites of individual patients at the same timepoint. The present study is also the first study to utilise freshly generated

anolyte solution to disinfect debrided DFU tissue, enabling the investigation of a potential novel treatment alternative for DFUIs and circumventing many of the confounders associated with comparative wound treatment-based clinical studies.

6.1. The prevalence of distinct staphylococcal species differs significantly between people with diabetes with and without DFUs

The prevalence of distinct staphylococcal species was investigated in 76 patients with and without DFUs, shortly after the time of sample collection. The DFU participant group was predominantly male (67/76 [88.2%]), and the prevalence of each distinct ulcer type (neuropathic [77.6%], neuroischaemic (18.4%], and ischaemic [3.9%]) correlated with previous studies (344-349). The indicators for oral health status were similar overall amongst both participant groups, however the average plaque indices were higher in patients with DFUs (*N*=72, range 0-100) than those without DFUs (*N*=61, range 0-100) and this group also reported less frequent toothbrushing (54/76 [71.1%] once per day or less). The time period since participants most recent dental appointments were similar; the majority of participants in both patient cohorts (51/76 [67.1%]) reported that their most recent dental visit was >12 months prior to sampling (Table 4.2), however this may have been influenced by COVID-19 pandemic restrictions.

Staphylococcal species were recovered from DFU swabs of 73/76 (91%) study participants. In Western countries, *S. aureus* is consistently the predominant pathogen isolated from DFUs and DFUIs (98, 142, 290, 327, 343). In agreement with these previous findings, *S. aureus* was the most prevalent species recovered from DFUs (36/76 [47.4%]) in the present study (Table 4.6), although it was commonly recovered alongside other CoNS species (16/76 [21.1%]) (Table 4.6). The majority of the DFUs sampled in the present investigation (53/76 [69.7%]) were superficial and likely harboured distinct microbial communities to those of chronic, more progressive DFUs. Staphylococci have been reported as being more prevalent and likely to cause infection in superficial wounds, while *S. aureus* (and MRSA) and the Gram-negative pathogen *P. aeruginosa* have been reported in deeper, bone-deep ulcers (350). *Staphylococcus epidermidis* was also identified in the DFUs of this patient cohort, however this species was not highly prevalent (10/76, 13.2%) at this anatomical site, despite being identified in the oro-nasal cavity of all 76/76 (100%) patients with DFUs. However, although not as prevalent in DFUs as *S. aureus*, further study of the role of *S. epidermidis* in DFUI aetiology is important, due to the ability of this species to form chronic and often antibiotic recalcitrant infections (351).

Nasal colonisation by *S. aureus* has long been considered an endogenous risk factor for systemic infections and bacteraemia (272, 273, 352-354), potentially resulting in the establishment of infection at distant anatomical sites and wounds via the vasculature and/or by direct hand transfer. A high rate of nasal carriage of *S. aureus* has been reported in people with diabetes (98, 99, 255, 281, 353), and Dunyach-Remy *et al.* (2017) identified the nares as a source for *S. aureus*-infected DFUs using DNA microarray profiling (98). However, these researchers did not investigate *S. epidermidis* populations in the same patient cohort. *Staphylococcus aureus* was significantly more prevalent ($P<0.01$) in oro-nasal sites of 60/76 (78.9%) patients with DFUs than in the corresponding sites of 31/76 (40.7%) patients without DFUs. The anterior nares was the site most frequently colonised by *S. aureus* in patients with DFUs (37/76 [48.6%]). *Staphylococcus aureus* was significantly more prevalent across all antomical sites investigated in patients with DFUs than in participants without DFUs ($P<0.05$) (Table 4.6), correlating with previous pilot research (343). This might indicate a bi-directional relationship between DFUs and oro-nasal carriage of *S. aureus*, perhaps influenced by freuqent hospital visits as a result of DFU treatment requirements.

Staphylococcus epidermidis was more prevalent across all anatomical sites in patients with DFUs than in participants without DFUs (Table 4.6). It was significantly more prevalent in periodontal pockets ($P<0.01$) and on the finger skin ($P<0.05$) of patients with DFUs than those without DFUs. It was likely more prevalent on the finger skin in this patient cohort due to frequent hospital exposure and their frequent close proximity to other patients with DFUs. *Staphylococcus epidermidis* was most frequently identified from the oral cavity (50/76 [65.8%]), particularly the periodontal pockets (34/54 [62.9%]) rather than the nares (45/76 [59.2%]) in patients with DFUs, indicating preferential oral rather than nasal colonisation by *S. epidermidis*. In participants without DFUs, *S. epidermidis* was most frequently recovered from the nares (39/76 [51.3%]) and was significantly less prevalent in periodontal pockets (27/76 [35.5%]) than those with DFUs ($P<0.01$), further suggesting that the presence of a DFU influences staphylococcal populations in people with diabetes.

The present study was significantly impacted by the COVID-19 pandemic, during which people were far more conscious of respiratory and hand hygiene, particularly in healthcare settings. It is possible that microorganisms present on hands were killed on patient presentation at the diabetes clinic via hand sanititiser. Mask wearing may also have had an effect on the study outcomes to some degree, where there may have been an increased amount of face touching, associated with removing and adjusting face masks, particularly when communicating with podiatry team members during foot care appointments. The

COVID-19 pandemic is also likely to have influenced the attendance of participants at routine dental and foot care appointments, particularly as people with diabetes were considered particularly vulnerable to developing severe manifestations of COVID-19 and as such, were advised to cocoon at several stages during the pandemic.

6.2. The oro-nasal cavity is an endogenous reservoir for DFUIs

The identification of the same species in the oro-nasal cavity and DFUs of 36/76 (47.3%) patients with DFUs (26/76 [34.2%] *S. aureus*, 10/76 [13.2%%] *S. epidermidis*) indicated a possible endogenous microbial reservoir that could only be confirmed by genetic comparison of staphylococcal isolates from both anatomical sites. High-throughput WGS platforms and bioinformative typing tools such as cgMLST and wgMLST provide a time-efficient and cost-effective informative approach for discriminatory bacterial strain comparisons in both closely related and highly diverse populations (355). In the present study, identical *S. aureus* STs were identified from the oro-nasal and ulcer sites of the majority of participants 19/26 (73.1%) from whom *S. aureus* was recovered from both sites (Fig. 5.5), providing evidence for the trafficking of these organisms between sites as discussed previously (99). The primary oro-nasal site site from where closely-related DFU isolates were identified was the nares, and closely related nasal and DFU isolates were recovered from 15/26 (57.7%) of participants. This consolidates previous evidence that trafficking likely occurs from the nares to other anatomical sites (36, 99, 262, 318, 356).

A comparatively more diverse population structure was evident amongst *S. epidermidis* isolates recovered from both participant groups. For the majority of participants (*N*=32 participants with DFUs, *N*=19 participants without DFUs) multiple distinct *S. epidermidis* STs were detected from the same participant, from distinct anatomical sites as well as from the same site (Figs. 5.3 and 5.4). Despite the high level of population diversity for this species, isolates recovered from four of the 10 (40.0%) patients from whom *S. epidermidis* was recovered from both oro-nasal and ulcer sites were identified as the same ST (Table 5.8). Identical STs from DFUs were identified in the oral cavity (*N*=2) and periodontal pockets (*N*=2), while two patients also harboured closely related nasal and DFU STs. Due to the prevalence data, it is likely that trafficking of *S. epidermidis* to the nares from the oral cavity is more likely, and that the oral cavity is a more likely reservoir for *S. epidermidis*-DFUIs. These findings suggest that the potential role of *S. epidermidis* in DFUs should not be overlooked in future studies.

Periodontal pockets harbour a diverse array of periodontal pathogens that have been reported in the bloodstream following toothbrushing, resulting in transient bacteraemia

(357). Lucas *et al.* reported the most prevalent bacteria in blood culture after toothbrushing were *S. epidermidis* and *S. warneri*, which were significantly more prevalent than in pre-toothbrushing blood culture samples (357). It would be interesting to investigate whether periodontal pocket carriage of *S. epidermidis* is associated with higher rates of transient *S. epidermidis* bacteraemia following toothbrushing and DFUI using wgMLST. Due to the high oral prevalence of *S. epidermidis* in patients with PD (48) or diabetes (99) it is plausible that the oral cavity constitutes an endogenous reservoir for *S. epidermidis*-causing DFUIs. It would be interesting to investigate whether *S. aureus*- or *S. epidermidis*-transient bacteraemia originating from the oro-nasal cavity is a potential risk factor for DFUIs. This study is the first to investigate oro-nasal staphylococci and DFUs, however it could be built upon by ruling out bloodstream transmission, utilising WGS technology to replicate the present study in a similar manner. Oral rinse, periodontal pocket samplings, DFU swabs and blood samples would be required to identify whether this is the case, and a pilot investigation could be carried out to investigate whether this is a possibility. Based on data outlined in the present study, translocation of oro-nasal strains to distant sites on account of periodontal disease and diabetes is probable. A schematic overview is outlined Fig. 6.1. However, other mechanisms for translocation should be investigated in the future, sucha as bloodstream transmission as discussed to determine the most plausible method of transfer.

The results of this investigation provide valuable evidence for potential clinical interventions that may reduce the risk of DFUIs, and subsequently osteomyelitis a severe complication associated with DFUIs, considerably. Screening procedures of the nares of patients with DFUs should be commonplace to identify if people are carriers of *S. aureus*, for those at risk to be aware of potential contamination and infection arising from their nose.

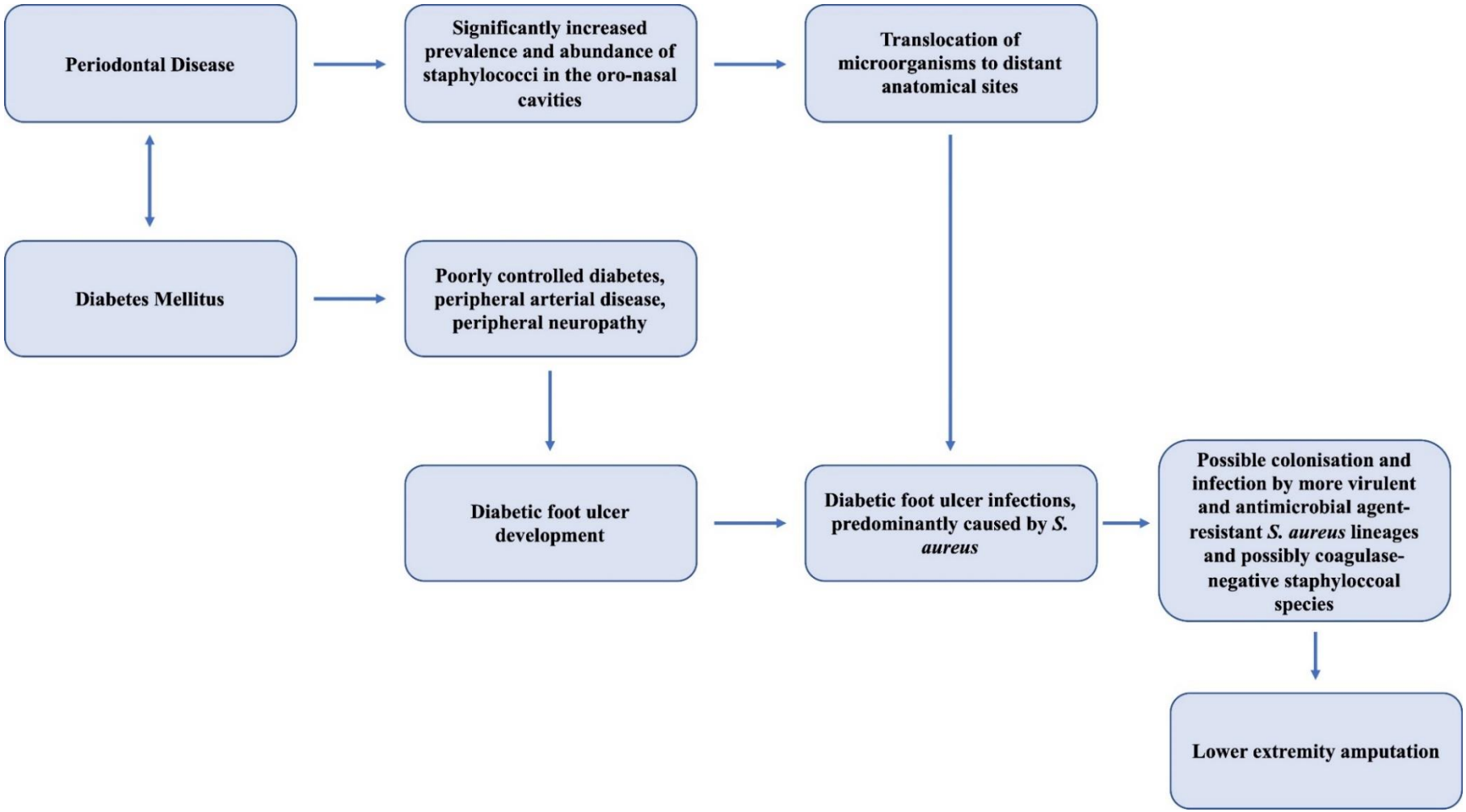

Periodontal Disease
Significantly increased prevalence and abundance of staphylococci in the oro-nasal cavities
Translocation of microorganisms to distant anatomical sites
Diabetes Mellitus
Poorly controlled diabetes, peripheral arterial disease, peripheral neuropathy
Diabetic foot ulcer development
Diabetic foot ulcer infections, predominantly caused by S. aureus
Possible colonisation and infection by more virulent and antimicrobial agent-resistant S. aureus lineages and possibly coagulase-negative staphyloccoal species
Lower extremity amputation

Figure 6.1. Overview of the bi-directional relationship between diabetes and periodontal disease, and the translocation of oro-nasal staphylococci to diabetic foot ulcers resulting in colonisation and subsequent infection. Previous research from this laboratory and the findings of the present study showed that oro-nasal staphylococci are significantly more prevalent and abundant in people with periodontal disease (48). Periodontal disease is three-fold more prevalent in people with diabetes and has been reported to affect metabolic control (11), increasing the severity of the condition. Poor metabolic control in conjunction with deteriorating overall health and damage to the lower extremities can result in the development of chronic DFUs. Chronic, mismanaged DFUs can result in DFUIs, which are predominantly caused by *S. aureus*, and other staphylococcal species. Increased oral carriage of staphylococci, particularly *S. aureus* and *S. epidermidis* in patients with diabetes on account of periodontal disease indicates a possible endogenous risk factor for translocation of these organisms to DFUs. This can result in DFUIs that can give rise to osteomyelitis and subsequent lower extremity amputations.

Moreover, increased awareness of oral health practices and the application of a daily oral rinse to reduce the potentially harmful microbial bioburden in the mouth and periodontium, could be implemented to reduce the possibility of the trafficking of oral microorganisms to at-risk sites. Chrlorohexidine mouthwash is effective in reducing microbial bioburden in the oral cavity (358, 359), and has been reported to reduce the severity of PD (360). Furthermore, topical decolonisation agents such as nasal mupirocin could also be used (361), which has been shown to be highly effective in reducing nasal *S. aureus* populations (361, 362). Oral and nasal decolonisation should be implemented in conjunction with one another to decolonise the oro-nasal cavity, reducing the possibility of trafficking within the oro-nasal cavity and to other anatomical sites.

It is essential that dental practitioners and diabetes-management clinicians work in tandem to emphasise the importance of oral health to people with diabetes. Oral health has unfortunately been widely overlooked in diabetes management in clinical settings. Hyperglycaemia in diabetes is associated with the development of oral complications including periodontal disease, xerostomia, oral candidiasis and burning mouth sensory disorder (363). Ahern *et al.* (2021) reported that in the the American Diabetes Association's "Standards of Medical Care in Diabetes", oral health examinations are recommended, however this is not commonplace in Ireland (363). The oral health status of the participants in the present investigation was sub-optimal, with the majority of participants reporting brushing their teeth once a day or less, did not practice interdental care and did not visit the dentist annually. Bleeding on probing was observed in the majority of participants who underwent probing (37/54 [68.5%]), a clear indicator of PD (Table 4.2). Moreover, both participant cohorts had an average of 15 natural teeth out of a possible 32, providing further evidence for poor oral hygiene practices and tooth loss (Table 4.2). The integration of routine dental checkups and oral hygiene activities into routine diabetes care pathways would provide multiple benefits for patients with diabetes. These include, improving quality of life, reducing the incidences of oral pain and tooth loss and therefore also positively influencing their abilities to sustain a healthy, nutritious diet, which would also positively influence diabetes management overall. In addition, dental professionals are especially equipped to identify risk factors and oral signs of poor metabolic control, such as xerostomia, PD and oral candidiasis. Oral health practicioners could recommend and motivate behavioural changes in oral health in people with diabetes to aid in the medical aspect to improve their overall health. Due to the identification of the oro-nasal cavity as an endogenous reservoir for DFUIs, it would be beneficial to employ a dental hygienist on site in Diabetes Centres. This may improve the oral hygiene of patients attending diabetes clinics, and provide routine

dental care treatments and advise patients on the importance of oral health in managing their diabetes, possibly preventing more serious diabetes-related complications. In an ideal world this would be a straightforward procedure, however it is not a certainty that everyone will abide by advice given by oral health practitioners. Thus, patients who are committed to improving their health would benefit the most from decolonisation procedures, and oral health advice and practices aimed at improving their wellbeing.

A systematic investigation involving patients with type II diabetes would be interesting to investigate whether the application of good oral health and awareness practices improves the quality of life for these patients. It should be possible to identify whether good oral hygiene practices in people with DFUs are associated with a decreased incidence of diabetes-related complications, including DFUIs. An on-site dental hygienist could also determine if there has been a reduction in the severity of gingivitis or PD in these patients, which could reduce oral staphylococci and reduce the risk for an endogenous source of DFUIs.

Likely patient-patient transmission of *S. aureus* was identified in 25 separate patients with DFUs (Fig. 5.2) who attended the clinic between a minimum of two days and 54 weeks apart 2020-2022. More surprising still was the identification of likely patient-patient transmission of *S. epidermidis*, which occurred on 10 separate occasions involving 25 patients both with and without DFUs, with an average of 3.4 allelic differences (range 0-15). This transmission occurred between a minimum two and a maximum of 58 weeks between clinical attendees between 2020-2022 (Figs. 5.3 and 5.4). However, this long timeframe does not support direct inter patient-transmission, as no patients were sampled in the clinic on the same day or week in any case. It is likely that this transmission could occur via contaminants (e.g., in dust particulates in the air or on surfaces in treatment rooms) where patients unknowingly acquired the bacteria, resulting in colonisation. Staphylococci can survive in the environment on dry surfaces for months (364), and colonisation may also have occurred via contaminated medical devices in the clinic. It would be interesting to investigate multiple anatomical sites of every patient entering the clinic over the course of one week, as well as sampling clinical surface areas to identify the prevalence of certain colonising strains of both *S. aureus* and *S. epidermidis*. Patients attended the clinic each week but only participated in the study once. Thus, patients could be in close contact in waiting areas or in the foot treatment clinic, however data for this regarding the possibility for a patient-patient transmission investigation was not available.

6.3. Anolyte is highly effective at reducing microbial bioburden in debrided DFU tissue

Research in the present study also moved towards identifying a novel, non-toxic and inexpensive treatment option to prevent serious infections. Previous studies using anolyte as a potential approach to treat chronic wounds had certain limitations, including anolyte storage conditions and pH, leading to less conclusive results. In the present study, the use of freshly generated anolyte at 200 ppm was shown to be highly effective in reducing the microbial bioburden in the debrided DFU tissue samples investigated, using both 1 ml and 10 ml volumes (Tables 3.2 and 3.3, Fig. 3.3). The fold-reductions in tissue microbial loads identified from tissue samples treated with 1 ml of 200 ppm anolyte incubated aerobically, anaerobically, and aerobically on Sa*Select*™ were significant ($P<0.01$), and 10 ml further significantly reduced the microbial bioburden ($P<0.01$) (Table 3.2). This study is the first study to use debrided tissue rather than direct wound treatment, allowing for a circumvention of patient specific healing capabilities, including haematosis, blood glucose levels, blood pressure, tissue perfusion, inflammation, granulation and epitheliazation capabilities (365), and disease severity variables, which did not influence the results in the present study. The design of this study, utilising freshly generated anolyte and debrided DFU tissue, might be useful for other similar studies in the future investigating DFUs, or other chronic wounds, treatment alternatives in a non-clinical setting.

It has been previously documented that anolyte is more effective, more microbicidal and less toxic to healthy tissues than topical antiseptics, including hydrogen peroxide and povidone iodine, which are known to inhibit growth and proliferation of fibroblasts around the ulcer, resulting in reduced healing time (173, 366). It is possible that an increased concentration of anolyte or repetitive immersion treatments would further decrease the microbial content within ulcers and infected ulcers, without toxic side-effects. Further study with an increased number of patients with clinical signs of infection at ulcer sites would be necessary to investigate this hypothesis further.

The results of the present study provide promising results for possible further clinical interventions and clinical trials. For a clinical investigation, a much larger volume of anolyte or a stream application may be beneficial to apply the anolyte. This is due to the much larger surface area of the foot, as well as antioxidant enzymes present in live host tissue which would quickly neutralise a small volume of anolyte. Using a large enough volume, or constant stream application of freshly generated anolyte, it would be straightforward and inexpensive to use this technology to significantly disinfect active DFUs and increase wound healing times. Previous investigations of this nature have utilised bottled anolyte, which is not metastable, and storage conditions and pH have varied. Recently,

anolyte has been shown to be held in a metastable state for several days if kept in a light shaded or brown bottle (367, 368). Anolyte is not readily available, and a generator is required to produce fresh, pH neutral and metastable anolyte. Thus, anolyte preserved in brown or shaded bottles would enable patients with DFUs to apply anolyte to their DFUs during regular DFU cleaning routines at home. This would enable patients to significantly reduce the risk of developing a DFUI if a clinical trial investigation in the future reports freshly-generated, metastable anolyte is highly effective in disinfecting live DFUs in the clinic.

Following on from the present research, future investigations in clinical settings should use a large enough volume volume of freshly generated anolyte to immerse the foot of the patient with the DFU, as well as utlising a shower head type delivery device to wash the DFU with anolyte. It would be interesting to calculate microbial bioburens per DFU investigated pre- and post- washing with anolyte, and to identify if certain species present in the DFU appear more susceptible to the effects of the wash. Lastly, anolyte in the present investigation was maintained at 200 ppm to wash the debrided DFU tissue. Subsequent investigations could utilise increased concentrations (e.g., 500 ppm) of freshly generated anolyte to increase its overall effectiveness at a clinical level.

6.4. Conclusions

This study was the first study of its kind, highlighting a link between oro-nasal staphyloccal carriage and DFUIs. Staphylococcal populations were higher in prevalence and varied more in participants with DFUs than participants without DFUs. Both *S. aureus* and *S. epidermidis* were much more prevalent in the oro-nasal cavities of patients with DFUs than participants without DFUs. Oral health status and practices were sub-optimal in both participant cohorts, with minimal toothbrushing, interdental care and visits to oral health practictioners reported.

Staphylococcus aureus was the predominant organism recovered in the DFUs (38/76 [50%]) and was more prevalent in the nares (36/76 [47.4%]) than the oral cavity (35/76 [46.1%]) or periodontal pockets (13/54 [24.1%]) in patients with DFUs. *Staphylococcus epidermidis* predominated in the oral cavity (56/76 [73.6%]) and periodontal pockets (34/54 [62.9%]) and was also prevalent in the DFUs (10/76 [13.2%]) in the same patient cohort. Identical staphylococcal species were identified in the oro-nasal cavity and DFUs of 38/76 (50.0%) patients with DFUs, including 26/76 (34.2%) *S. aureus* and 10/76 (13.2%) *S. epidermidis*. Whole genome sequencing provided irrefutable evidence that *S. aureus* and *S. epidermidis* strains in oro-nasal and ulcer sites of 19/26 (73.1%) and 4/10 (40.0%) participants with DFUs, respectively, were highly-related indicative of transmission between endogenous sites. The high prevalence of both *S. aureus* and *S. epidermidis* at oro-nasal sites relative to other CoNS species in the present study highlights the importance of oral health interventions to reduce microbial bioburden and the likelihood of the oro-nasal cavity as an endogenous source of DFUIs. The nares appeared to be the likely source for trafficking of *S. aureus* between the oro-nasal cavity and DFUs and had the highest number of closely-related strains (15/26 [57.7%]) from the DFU. The oral cavity and periodontal pockets were the likely source for trafficking of *S. epidermidis* between oro-nasal and DFUs, indicating that periodontal disease is a risk factor for endogenous *S. epidermidis*-DFUIs.

The present study utilising anolyte as a disinfectant was the first study of its kind conducted on debrided DFU tissue. Anolyte at 200 ppm was shown to be highly effective in significantly reducing the microbial bioburden of aerobic, anaerobic and staphylococcal organisms from debrided DFU tissue. The increase of 1 ml to 10 ml of anolyte significantly reduced microbial bioburden further ($P<0.01$). Tissue samples immersed in 1 ml and 10 ml were reduced even below the suggested $>10^5$ CFU/g threshold for microbial infection, indicating an alternative clinical treatment option for DFUIs. Due to its effectiveness at reducing the microbial bioburden of debrided tissue, anolyte should be brought to a clinical trial study to disinfect active ulcers within the clinic. Based on the results of this novel study, it is critical that dental and oral health plays a more predominant role in diabetes

management, that the role of endogenous staphylococcal populations in people with diabetes is further investigated and includes specific research on the role of *S. epidermidis*, and the use of anolyte requires further investigation for its use in DFUI disinfection.

Future research should also investigate the role of the oro-nasal cavity in deeper chronic DFUs, and in cases of osteomyelitis, utilising bone debridement samples and swab samples to investigate whether oro-nasal microorganisms contribute to severe DFUIs. This could be undertaken by investigating the prevalence of both staphylococcal species and anaerobic organisms, which may be prevalent in the oral cavity and particularly in periodontal pockets, in deeper ulcers

Furthermore, the ability of anolyte to reduce the microbial bioburden in DFUs and DFUIs in both neuropathic and deeper chronic ulcers should be assessed in a clinical setting, initially using the parameters developed in the present study (i.e. freshly generated, consistent concentration in ppm and metastable). This future work could also investigate the potential of anolyte to reduce the bioburden of resident microorganisms causing infections in deeper wounds compared to surface level ulcers and wound healing. The potential use of anolyte to treat bone deep ulcers should also be considered, which potentially could prevent the development of osteomyelitis, preventing possible lower extremity amputation.

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Appendix 1

Investigation of Anolyte Solution for the Treatment of Diabetic Foot Ulcer Infections

Project Overview

Staphylococcal bacteria are the predominant cause of diabetic foot ulcer infections (DFUIs) in patients with Type 2 Diabetes. This project focuses on the disinfection of debrided DFUI tissue using a superoxidised anolyte solution. Based on a dilute saline solution, anolyte is highly microbiocidal, non-toxic, pH – neutral, odourless and environmentally friendly. The aim of this study is to determine the optimal conditions for complete disinfection of debrided DFUI tissue as a means for informing future, intervention-based studies.

Who is suitable to participate?

Participation is completely voluntary and all information will be kept strictly confidential.

Suitable participants must:

1. Be over 18 years of age
2. Be capable of providing informed consent
3. Be undergoing routine clinical debridement therapy in the DDC foot clinic
4. Patients are NOT excluded from the study on the basis of antibiotic usage

What does participation involve?

Participants will undergo routine debridement therapy in the DDC foot clinic as normal. Relevant medical history and recent antibiotic usage will be recorded. Some of the debrided tissue will be retained and transported to the Dublin Dental University Hospital Microbiology Research Unit for microbial disinfection and analysis.

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Appendix 2

Exploring the oro-nasal cavity as a potential microbial reservoir for diabetic foot infection

Project Overview

Staphylococcal bacteria are prevalent, opportunistic pathogens harboured in the oro-nasal cavities particularly in those with gum disease. This study will focus on the species distribution, prevalence, abundance and types of staphylococcus in the mouth/nose/ulcers of patients with type II diabetes to determine if the nose/mouth can be a source for diabetic foot ulcer infection (DFUI). If so, oro-nasal decolonisation strategies may be useful in DFUI prevention.

Who is suitable to participate?

Participants will have either 1) active foot disease or 2) no history or risk factors for foot ulcer development.

Suitable Participants must:

1. Be over 18 years of age
2. Be capable of providing informed consent
3. Have at least one natural tooth
4. Have no underlying heart conditions such as cardiovascular disease
5. Have no other serious underlying conditions apart from Type 2 Diabetes
6. Patients are NOT excluded from the study on the basis of antibiotic usage

What does participation involve?

Participation is completely voluntary and all information will be kept strictly confidential.

- The participant will be asked some questions regarding general health and recent relevant medical history and will be asked to rinse sterile water around their mouths for 1 minute (oral rinse).
- Sterile swabs will be used to sample the following sites of each participant: the nose, the index finger, a toe and an ulcer (if present).
- A complete periodontal examination will be undertaken and some absorbent paper strips will be used to collect fluid from between the teeth and gums (gingival crevice).

The whole process will take a maximum of 45 minutes to complete and will be undertaken at the Diabetes Day Centre (DDC) a time that is convenient to the participant. The sampling process will be undertaken by a dental hygienist from the Dublin Dental University Hospital, and podiatrists from the DDC foot clinic.

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Immersion of debrided diabetic foot ulcer tissue in electrochemically generated pH neutral hypochlorous acid significantly reduces the microbial bioburden: whole-genome sequencing of *Staphylococcus aureus*, the most prevalent species recovered

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SUMMARY

Background: Diabetic foot ulcer infections (DFUIs) are the leading cause of lower-limb amputations, mediated predominantly by *Staphylococcus aureus*. pH-neutral electrochemically generated hypochlorous acid (anolyte) is a non-toxic, microbiocidal agent with significant potential for wound disinfection.
Aims: To investigate both the effectiveness of anolyte for microbial bioburden reduction in debrided ulcer tissues and the population of resident *S. aureus*.
Methods: Fifty-one debrided tissues from 30 people with type II diabetes were aliquoted by wet weight and immersed in 1- or 10-mL volumes of anolyte (200 parts per million) or saline for 3 min. Microbial loads recovered were determined in colony forming units/g (cfu/g) of tissue following aerobic, anaerobic and staphylococcal-selective culture. Bacterial species were identified and 50 *S. aureus* isolates from 30 tissues underwent whole-genome sequencing (WGS).
Findings: The ulcers were predominantly superficial, lacking signs of infection (39/51, 76.5%). Of the 42/51 saline-treated tissues yielding $\geq 10^5$ cfu/g, a microbial threshold reported to impede wound-healing, only 4/42 (9.5%) were clinically diagnosed DFUIs. Microbial loads from anolyte-treated tissues were significantly lower than saline-treated tissues using 1 mL (1065-fold, 2.0 log) and 10 mL (8216-fold, 2.1 log) immersion volumes ($P<0.0005$). *S. aureus* was the predominant species recovered (44/51, 86.3%) and 50

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isolates underwent WGS. All were meticillin susceptible and comprised 12 sequence types (STs), predominantly ST1, ST5 and ST15. Whole-genome multi-locus sequence typing identified three clusters of closely related isolates from 10 patients indicating inter-patient transmission.

Conclusions: Short immersions of debrided ulcer tissue in anolyte significantly reduced microbial bioburden: a potential novel DFUI treatment.

Introduction

Diabetes mellitus affects 537 million people worldwide [1], up to 25% of whom are likely to be affected by debilitating and costly diabetic foot ulcers (DFUs) during their lifetimes [2]. In 2019, Kerr *et al.* reported that DFU-related care accounted for more than 90% of the £837—962 million annual diabetes-related expenditure in England [3]. At least 7000 people with diabetes in England undergo lower extremity amputation (LEA) annually [4] with devastating consequences for quality of life, risk of death within the following five years and significantly further increasing the cost of hospital care [5]. Annual expenditure for post LEA care has been reported to be £20.81 million [3]. Recent accurate corresponding Irish LEA expenditure is not readily available due to the lack of a diabetes register; however, diabetes-related care has previously been estimated to account for 4—6% of the national healthcare budget [6].

Approximately 50—60% of DFUs [7] develop DFU infection (DFUI), increasing the risk of hospitalization and LEA by 56- and 155-fold, respectively [8]. Previous studies reported that a DFU microbial bioburden of $\geq 10^5$ colony-forming units (cfu) impedes wound healing [9]. Around 20% of DFUIs result in LEA [10]. *Staphylococcus aureus*, the leading cause of skin and soft tissue infections, is the most frequently isolated bacterial species from DFUIs, of which approximately 18% are meticillin-resistant *S. aureus* (MRSA) [11]. Targeted amplicon sequencing has revealed that DFUIs are often polymicrobial, and specific microbial population compositions influence treatment outcomes [12—14].

Electrochemically generated (ECA) solutions are produced by passing a dilute brine solution through an electric field in an electrolytic cell and segregating the two oppositely charged solutions created termed anolyte and catholyte. Anolyte is a highly positively charged aqueous solution containing predominantly hypochlorous acid which has been purified of negative ions by an ion-selective membrane. Not all 'anolyte' ECA solutions come from a process such as this and may not include membrane separation, and are more akin to hypochlorite solutions. Some are available in bottled form. Hypochlorous acid is a powerful antimicrobial oxidant that is naturally produced by neutrophils. A comprehensive review of the antimicrobial efficacy and mode of action of hypochlorous acid and its use for therapeutic support of infections was published recently [15].

Modern ECA generators can be configured to produce pH-neutral anolyte solutions that are environmentally friendly, non-toxic, inexpensive, highly microbicidal, sporicidal and capable of penetrating biofilms [16—18]. Hypochlorous acid has been shown to decrease the bacterial bioburden and promote wound and burn healing by repetitive irrigation [19—21]. In addition, anolyte-soaked wound dressings and sprays have shown promise in post-surgical management of DFUIs, promoting healing, reducing bacterial load, odour and re-infection rates [22,23].

As kidney disease and ischaemia are common diabetes-related complications which limit the success of systemically distributed antibiotics, the use of alternative inexpensive, topical disinfectants has significant potential benefits. The current study sought to investigate the consistent effectiveness of pH-neutral anolyte at reducing bioburden in debrided DFU tissue, to investigate the range of microbial species present, and to analyse the population of the most prevalent species, *S. aureus*, by whole-genome sequencing (WGS).

Methods

Patient recruitment

Consenting patients with type II diabetes undergoing routine DFU debridement at the St James's Hospital Dublin (SJH) foot clinic were invited to participate. Ethical approval was granted by the SJH and Tallaght University Hospital Joint Research Ethics Committee (Approval No. 2019-12). Multiple debrided tissue samples collected from the same individuals were collected a minimum of two weeks apart.

Ulcer examination and debrided ulcer tissue collection

A specialist clinical podiatrist performed a brief ulcer assessment using the Texas wound classification scheme [24], recorded any prior DFU-related interventions and collected debrided tissues.

Anolyte

Anolyte solution was produced by electrochemical activation of a NaCl solution using a Qlean-Genie™ UL-75a ECA generator (Qlean Tech Enterprises, MN, USA) [25,26]. The generator was configured to produce anolyte measured at 800 parts per million (ppm) free available chlorine (FAC) at pH 7.0, having an oxidation—reduction potential (ORP) of +880 mV and consisting of approximately 632 ppm hypochlorous acid (HOCl) (79%) and 162 ppm hypochlorite ion (OCl^-) (20.2%). The FAC concentration of freshly generated anolyte was determined using a Hach colorimeter (Hach, Western Industrial Estate, Dublin, Ireland) and adjusted to 200 ppm in sterile water (Milli-Q®, Merck, Cork, Republic of Ireland) prior to disinfection of tissue samples. Under the EU Biocides Regulation 528/2012 [27] and Commission Implementing Regulation (EU) 2021/364 of 26 February 2021 [28], the anolyte solution used in this study would be classed as active chlorine generated

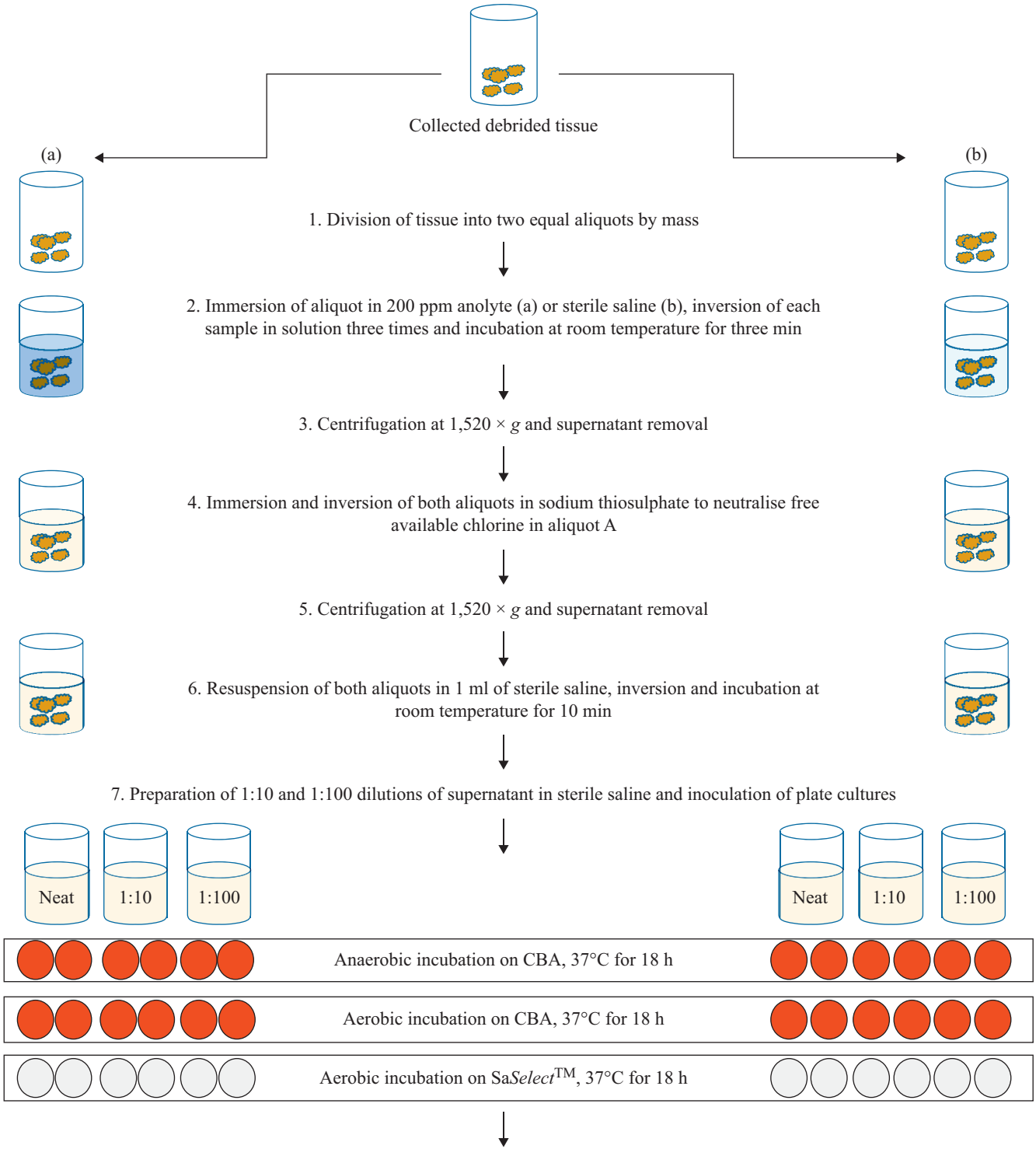

Figure 1. Comparative immersion treatment and microbiological analyses of debrided tissue aliquots in (a) 200 ppm anolyte or (b) sterile saline. Debrided tissues were divided into two aliquots based on wet weight which ranged from 35 to 75 mg. One aliquot was immersed in freshly generated 200 ppm anolyte, the other in an equal volume of sterile saline. Each tube was inverted three times to maximize contact between the tissue and the treatment liquid and was incubated at room temperature (RT) for 3 min. Following incubation, each sample was subjected to centrifugation at 1520 × *g* (Centrifuge 5430, Eppendorf, Hamburg, Germany) for 2 min. The supernatant was discarded, and the anolyte/saline treatment step was repeated three times. Following the third treatment step, tissues were immersed in sodium thiosulphate (0.5% [w/v], inverted five times to mix and incubated at RT for 3 min to neutralize any remaining free available chlorine (FAC). Samples Ano1–Ano20 were immersed in 1 mL volumes of anolyte/saline and sodium thiosulphate, whereas samples Ano20–Ano51 were immersed in 10 mL treatment volumes. Following immersion in sodium thiosulphate, tissues were recovered by

from sodium chloride by electrolysis as an active substance for use in biocidal products of Product Type 1 – human hygiene.

Microbiological processing

Tissue samples were divided into two equal aliquots by wet weight (range 35–75 mg). Paired aliquots were immersed in equal volumes of either freshly generated anolyte or sterile saline for 3 min (Figure 1). Previous research revealed that the FAC in anolyte rapidly reduces upon contact with organic material [16] and, therefore, two distinct treatment volumes were investigated; 1 mL (Samples Ano1–Ano20) and 10 mL (Samples Ano21–Ano51) to determine whether an excess of anolyte further reduced microbial loads in tissues. Following immersion, tissues were recovered by centrifugation at 1520 × *g* (Eppendorf model 5430, Hamburg, Germany) and the supernatant discarded. Residual FAC was neutralized by immersion of tissues in 0.5% (w/v) sodium-thiosulphate, followed by washing and resuspension of tissues in sterile saline (Figure 1). Resuspended tissues were incubated in the final volume of saline for 10 min at room temperature with shaking at 200 rpm to release any remaining tissue-bound micro-organisms into the supernatant prior to microbiological culture (Figure 1).

Determination of microbial burden in tissue

An aliquot of supernatant from each tissue sample was diluted 10-fold in saline. Aliquots (100 μL) of each neat and diluted supernatant sample were used to inoculate duplicate Columbia blood agar (CBA) plates that were incubated at 37 °C in a static incubator under aerobic conditions, anaerobic conditions using a GasPak™ EZ anaerobic system (Fisher Scientific, Dublin, Ireland) and on staphylococcal-selective chromogenic Sa*Select*™ (Bio-Rad, Dublin, Ireland) agar (Figure 1). Following incubation, the cfu present on each plate were counted and residual microbial loads in tissues were determined as cfu per gram (cfu/g). Microbial load fold-reductions in anolyte-treated tissues relative to saline-treated tissues were calculated for all media and incubation conditions. A majority (42/51) of saline-treated tissues (considered as baseline microbial loads) yielded $\geq 10^5$ cfu/g (Table I, Supplementary Table S1); a microbial load threshold that has been previously reported to impede wound healing [9].

Isolate storage and identification

Isolates were stored at -80 °C on Microbank cryogenic bead vials (Pro-Lab Diagnostics, Birkenhead, UK). Staphylococcal species were presumptively identified based on colony colour on Sa*Select*™ medium. Definitive species identification was determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using the VITEK MS system (bioMérieux, Marcy L'Etoile, France) or Sanger-based sequencing of the 16S ribosomal RNA gene [29], which was carried out commercially (Source Bioscience, Cambridge, UK and Eurofins, Konstanz, Germany). Resultant 16S nucleotide sequences were subjected to BLAST-based identification (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

WGS analysis

Nucleic acid extraction and Illumina (Eindhoven, The Netherlands) short-read WGS was performed as previously described [30]. Short-read FASTQ files were assembled, quality assessed and subjected to whole-genome multi-locus typing (wgMLST) and minimum spanning tree (MST) construction using BioNumerics software (BioNumerics v8.0; Applied Maths, Sint-Martens-Latem, Belgium). Ridom SeqSphere+ software v7.0.4 (Ridom GmbH, Münster, Germany) was used to identify secreted protein A gene (*spa*) types, antimicrobial resistance and virulence genes from WGS datasets, which have been deposited in the NCBI Sequence Read Archive (BioProject PRJNA971107).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 25 (Chicago, IL, United States of America). Significant results were indicated by $P<0.05$.

Results

Types of ulcers sampled

DFU debrided tissue samples were collected from 30 participants with type II diabetes attending the foot clinic for routine debridement (Supplementary Table S1) over three years. Samples from patients who participated multiple times were collected a minimum of two weeks apart. Of the 51 samples recovered, 24 were collected from patients with a prior LEA history and 13 were from patients with more than one active ulcer (range two to four ulcers) at the time of sample collection. The average age of each sampled ulcer was 32 weeks (median 13, range 0–500 weeks). The ulcers sampled were predominantly neuropathic (42/51,82.4%), neuroischaemic (9/51,17.6%) and diagnosed as Texas wound grades 1A (39/51,76.5%); superficial and lacking clinical signs of infection. Four ulcers exhibited clinical DFUI symptoms, which were graded as 1B, 1D and 3D. Four of the ulcers sampled had penetrated tendon, capsule, bone or joint tissue and were graded as 2A ($N = 2$), 3C ($N = 1$) and 3D ($N = 1$).

Microbial load of tissues immersed in 1 mL volumes of saline or 200 ppm anolyte

The average (and median) bacterial loads recovered from 1 mL saline-treated tissues following aerobic and anaerobic culture on CBA and aerobic culture on Sa*Select*™ agar were $1.40 \times 10^6 \pm 5.28 \times 10^5$ (2.78×10^5), $9.23 \times 10^5 \pm 3.03 \times 10^5$

centrifugation as above, immersed in 1 mL sterile saline, inverted five times to mix and incubated at RT with shaking for 10 min. The supernatant from each tissue sample was subjected to two 10-fold dilutions from which 100 μL aliquots were used to inoculate duplicate Columbia blood agar plates that were incubated under aerobic conditions, anaerobic conditions and on the staphylococcal-selective chromogenic agar medium Sa*Select*™. Following incubation, the bacterial density in colony forming units (cfu) per g of starting tissue was recorded and the fold reduction in anolyte-treated tissue samples incubated under aerobic, anaerobic and staphylococcal-selective conditions was calculated and recorded.

Table I
Microbial recovery (colony forming units/g (cfu/g)) and fold reduction from debrided foot ulcer tissue after immersion in 1 mL or 10 mL volumes of sterile saline or 200 ppm anolyte following incubation under aerobic, anaerobic and staphylococcal-selective conditions

Treatment volume (samples)	1 mL (Ano 1–20)		10 mL (Ano 21–51)	
	Mean	Median[a]	Mean	Median[a]
Aerobic CBA (cfu/g)				
Saline (cfu/g)[b]	1.40E + 06	2.78E + 05	8.78E + 05	2.80E + 05
Anolyte (cfu/g)	5.11E + 04	8.1E + 03	2.86E + 04	2.30E + 03
Fold reduction	1513.4	61.6	12243.6	93.3
Log reduction	2.0	1.8	2.2	2.0
Anaerobic CBA (cfu/g)				
Saline (cfu/g)[b]	9.23E + 05	5.00E + 05	9.14E + 05	4.16E + 05
Anolyte (cfu/g)	2.49E + 04	7.83E + 03	4.13E + 04	1.48E + 03
Fold reduction[c]	74.7	44.1	2747.2	135
Log reduction	1.9	1.7	2.3	2.1
Aerobic Sa*Select*™ (cfu/g)				
Saline (cfu/g)[b]	6.29E + 05	3.20E + 05	1.67E + 06	2.97E + 05
Anolyte (cfu/g)	2.42E + 04	3.82E + 03	1.98E + 04	1.20E + 03
Fold reduction	1605.6	101.4	9656.9	145.1
Log reduction	2.2	2.1	2.5	2.2

CBA, Columbia blood agar.
[a] Wilcoxon Signed Rank Tests based on related samples deemed that the differences between the medians of saline- and anolyte-treated solutions was significant ($P<0.0005$) under all incubation conditions and treatment volumes investigated.
[b] Only 4/42 of the samples which yielded microbial loads $>10^5$ cfu/g, a threshold previously shown to impede wound healing [9], exhibited clinical signs of infection (Supplementary Table S1).
[c] Mann–Whitney U-test for independent samples indicated that the fold reduction distribution observed between anaerobically incubated samples treated in 1- or 10 mL-treatment volumes was significantly different ($P<0.05$) (Supplementary Figure S2).

(5.00×10^5) and $6.29 \times 10^5 \pm 2.38 \times 10^5$ (3.20×10^5) cfu/g of debrided tissue, respectively (Table I, Supplementary Table S2, Supplementary Figure S1). In contrast, the corresponding average (and median) bacterial loads recovered from 1 mL anolyte-treated tissues following aerobic and anaerobic culture on CBA and aerobic culture on Sa*Select*™ agar were $5.11 \times 10^4 \pm 3.54 \times 10^4$ (8.05×10^3), $2.49 \times 10^4 \pm 9.14 \times 10^3$ (7.83×10^3) and $2.42 \times 10^4 \pm 1.48 \times 10^4$ (3.82×10^3) cfu/g, respectively. Significant reductions (Z-3.92, $P<0.0005$) in the average bacterial load of 1 mL anolyte-treated tissues compared with l mL saline-treated tissues were observed following aerobic (2.0 log, 227.7–fold) and anaerobic (1.9 log, 74.7-fold) culture on CBA agar and aerobic culture (2.2 log, 1605.6-fold) on Sa*Select*™ agar (Table I).

Microbial load of tissues immersed in 10 mL volumes of saline or 200 ppm anolyte

As previous studies have shown that anolyte FAC decreases rapidly following contact with organic material [16], experiments were undertaken to investigate whether increasing saline and anolyte immersion volumes further reduced the microbial load of anolyte-treated tissues. Thirty-one tissue samples were subjected to immersion-treatment in 10 mL volumes of saline or anolyte, respectively. Following 10 mL saline-treatment, the average (and median) bacterial loads recovered following aerobic and anaerobic culture on CBA and aerobic culture on Sa*Select*™ agar were $8.78 \times 10^5 \pm 3.50 \times 10^5$ (2.80×10^5), $9.14 \times 10^5 \pm 4.68 \times 10^5$ (4.16×10^5) and $1.67 \times 10^6 \pm 6.79 \times 10^5$ (2.97×10^5) cfu/g, respectively. In contrast, the average (and median) bacterial loads recovered from 10 mL anolyte-treated tissues following aerobic and anaerobic culture on CBA and aerobic culture on Sa*Select*™ agar were $2.86 \times 10^4 \pm 2.11 \times 10^4$ (2.30×10^3), $4.13 \times 10^4 \pm 3.43 \times 10^4$ (1.48×10^3) and $1.98 \times 10^4 \pm 1.33 \times 10^4$ (1.20×10^5) cfu/g, respectively (Table I, Supplementary Table S2, Supplementary Figure S1). Significant (Z-4.86, $P<0.00001$) reductions in the average bacterial load (cfu/g) of 10 mL anolyte-treated samples compared with 10 mL saline-treated samples were observed following aerobic (2.2 log, 12,243.6-fold) and anaerobic (2.3 log, 2747.2-fold) culture on CBA, and aerobic culture (2.5 log, 9656.9-fold) on Sa*Select*™ agar (Table I, Supplementary Figure S1), respectively. Furthermore, the comparison of fold reductions observed between 1 mL and 10 mL treatment volumes revealed that, under anaerobic incubation conditions, the 10 mL treatment volume was significantly ($P<0.05$) more effective at reducing microbial bioburdens than the 1 mL treatment volume (Table I, Supplementary Table S2, Supplementary Figure S2).

Bacterial isolates identified from debrided DFU tissue

Definitive species identification was performed on 237 isolates recovered from saline-and anolyte-treated tissues under aerobic and anaerobic culture conditions. This ensured a widespread representation of isolates remaining in each sample, and that there was no species-enrichment in anolyte-treated samples. *Staphylococcus* was the most common genus identified (44/51, 86.3%), of which *S. aureus* was the most prevalent species recovered from tissue (30/51, 58.8%), followed by *Streptococcus* (9/51, 17.6%), *Proteus* (7/51, 13.7%), and *Enterococcus* (5/51, 9.8%) species. Twelve patients participated in the study on multiple occasions, with at least two weeks between each sample collection. Distinct microbial populations were detected from these samples on each occasion, with the exception of two samples collected from patient

P4 in December 2022 and January 2023, both of which exclusively yielded *P. mirabilis* (Supplementary Table S1).

WGS of S. aureus

As *S. aureus* was the predominant organism recovered from 30/51 (58.8%) distinct tissue samples, 50 *S. aureus* isolates selected as representatives of each distinct tissue sample were investigated by WGS. All isolates were meticillin-susceptible *S. aureus* (MSSA) and lacked the *mecA/C* genes. Other antimicrobial resistance genes detected included *blaZ* (33/50, 66.0%), *fosB* (34/50, 68.0%), *erm*(A) (4/50, 8.0%) and *qacC* (5/50, 10.0%). Genes encoding pore-forming toxins were detected in all 50 isolates, including the *hla* alpha-haemolysin gene (44/50, 88.0%) and the *luk*D/E leukocidin-encoding genes (35/50, 70%). The Panton–Valentine Leukocidin *lukF/S-PV* genes, the *edin* epidermal cell differentiation inhibitor toxin gene, and exfoliative toxin-encoding genes *etA/B/D* were not detected in any of the 50 isolates investigated. Immune evasion cluster (IEC) and enterotoxin genes including *sea/g/h/i/k/m/n/o/q/u* were detected in 41/50 and 49/50 isolates, respectively. The *tst1* gene encoding the toxic shock syndrome toxin was identified in 3/50 isolates (Supplementary Table S4).

Population analysis of S. aureus *using whole-genome multi-locus sequence typing (wgMLST)*

Fifteen distinct *spa* types and 12 distinct sequence types (STs) were identified, of which ST5 (t067, t002, t1082) ($N = 18$), ST1 (t127) ($N = 9$), and ST15 (t084) ($N = 6$) predominated (Supplementary Table S1). Isolates belonging to these STs have previously been described in oro-nasal and ulcer sites of people with diabetes in Ireland [31]. Of the seven patients from whom *S. aureus* was recovered on more than one occasion, persistent STs and *spa* types were identified in three patients (P20, P24, P29); isolates from each patient exhibited a maximum of four allelic differences (Figure 2). Multiple ST5 isolates with distinct *spa* types were recovered from two patients <4 months apart (Supplementary Table S4). The ST5-t002 strains originally recovered from Patient 17 were subsequently replaced by ST5-t067 strains (separated from t002 isolates by 270 wgMLST allelic differences). Patient 18 yielded ST5-t067, ST5-t1082 and ST5-t067 strains on the first, second and third samplings, respectively, indicative of persistent co-colonization with multiple distinct strains (>396 wgMLST allelic differences). Strain replacement was also observed in two patients; P4 (ST30 to ST15) and P19 (ST22 to ST1) from whom isolates were recovered >24 months apart (Supplementary Table S4).

A wgMLST-based MST revealed the presence of three distinct clusters of closely related isolates, within which neighbouring isolates were separated from each other by a maximum of three allelic differences (Figure 2), considerably below the previously proposed ≤24 allelic difference threshold for determination of isolate close relatedness [32]. Cluster 1 comprised ST5, t067 isolates ($N = 11$) from five patients, Cluster 2 comprised ST5, t1082 isolates ($N = 5$) from three patients and Cluster 3 comprised ST1, t127 isolates ($N = 6$) from two patients. All of the isolates within these three clusters were recovered within the same four-month period. Four isolates recovered from Patient P24 were separated from each other by a maximum of two allelic differences. One isolate recovered from P4 (Ano45-2) was separated from the most closely related isolate (Ano24-1) from P24 by only two allelic differences (Figure 2). These findings indicate transmission of specific strains of *S. aureus* among some of the study patients.

Association of microbial loads with clinical ulcer features

Four ulcers exhibited clinical signs of infection and yielded $\geq 10^5$ cfu/g, a microbial threshold previously associated with impeded wound healing [9]; however, an additional 38/51 (74.5%) saline-treated samples also yielded $\geq 10^5$ cfu/g (Supplementary Table S1), despite lacking clinical signs of infection. The prevalence of *S. aureus* and microbial load distribution of saline-treated samples incubated under each of the three culture conditions was compared based on the following clinical criteria: clinical signs of infection, neuropathic or neuroischaemic ulcers, distinct Texas classifications, number of active ulcers, age of oldest ulcer and prior LEA history. Of these criteria, the prevalence of *S. aureus* and the average and median microbial load in anaerobically incubated saline-treated samples was significantly higher ($P<0.05$) in patients with LEA history (Supplementary Table S3).

Discussion

According to the Irish Health Service Executive (HSE), 1948 people with diabetes required DFU-related in-patient hospital treatment in 2015, spending on average 14.5 days in hospital and 451 LEAs were performed [33]. In 2018, 40 people were admitted to a Dublin-based hospital for DFU-related treatment, staying an average of 31 days at a minimum cost of €25,000 each, excluding the considerable costs associated with LEA, laboratory tests, antibiotics and follow-up care. A simple, inexpensive and highly effective novel DFUI treatment could potentially reduce DFUI-associated consequences considerably.

Dublin Dental University Hospital water networks have been successfully maintained to an extremely high microbiological standard by residual treatment with 2 ppm anolyte for almost two decades [16,18]. Furthermore, research from this institution has demonstrated the safety of ECA solutions on reconstituted human oral epithelial models [16] and their effectiveness at significantly reducing biofilm formation in dental unit waterlines, washbasin U-bend traps and wastewater pipes [16,18,25,26,34,35].

Many previous studies reported the superiority of ECA solutions for wound disinfection and healing [19–23,36–39], however, these studies were predominantly clinically based, comparing healing outcomes between patients treated with ECA or placebo solutions. Variability including clinical criteria for defining DFUIs, antibiotic usage, patient-specific immune responses and healing abilities, likely confound the results of these studies. In addition, the solution generation conditions, composition, pH, storage times and conditions and the exact equipment involved in making the activated solution is lacking in many other publications makes it difficult to assess or compare efficacy.

Although relatively small, our study demonstrated significant microbial load reductions in *ex vivo* debrided DFU tissue following immersion in 200 ppm pH 7.0 anolyte, and thus

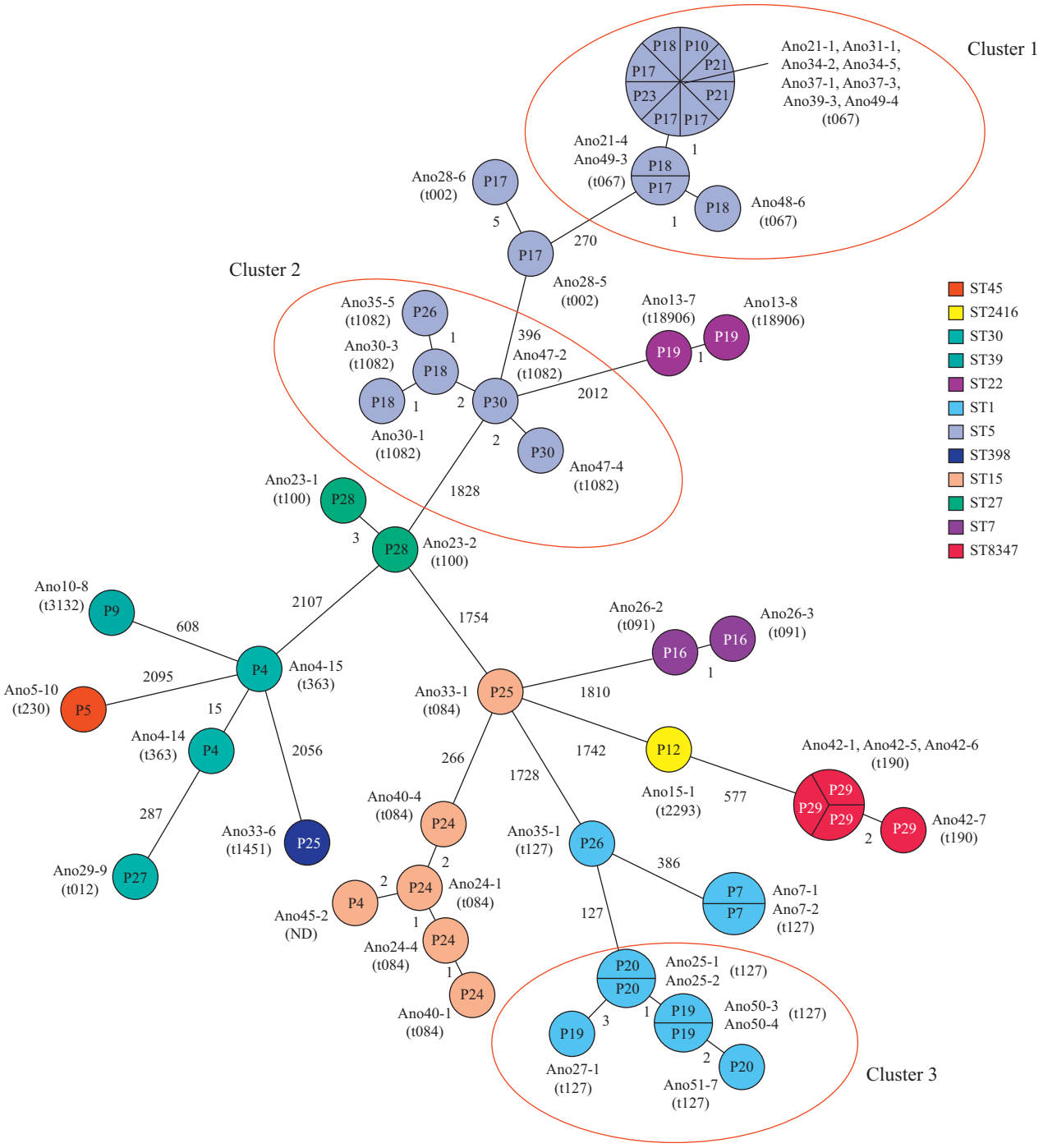

Figure 2. Minimum spanning tree based on whole-genome multi-locus sequence typing (wgMLST) of 50 *S. aureus* isolates from 30 debrided tissue samples from 20 patients. Construction of the minimum spanning tree (MST) was performed based on wgMLST using Bionumerics software (BioNumerics v8.0; Applied Maths, Sint—Martens—Latem, Belgium). Distinct patients are indicated by corresponding identifiers within each node of the MST. Node colours indicate 12 distinct sequence types (STs) according to the legend. Labels beside each distinct node indicate the name and *spa* type of each isolate investigated. Integers above branches indicate the number of allelic differences between neighbouring isolates. Three distinct clusters are apparent within the MST and are encircled in red. Cluster 1 comprises ST5, t067 isolates recovered from five separate patients (P10, P17, P18, P21, P23) over the same four-month period in which neighbouring isolates are separated by a maximum of one allelic difference. Cluster 2 comprises ST5, t1082 isolates recovered from three distinct patients (P18, P26 and P30) over the same one-month period in which neighbouring isolates are separated by a maximum of two allelic differences. Cluster 3 comprises ST1, t127 isolates recovered from two separate patients (P19 and P20) over a four-month period within which neighbouring isolates are separated by a maximum of three allelic differences.

patient-specific immunity, treatments and healing capabilities did not influence the study outcome. Significant microbial bioburden reductions were observed in both 1 mL and 10 mL anolyte-treated tissues relative to saline under all culture conditions. However, increasing the immersion volumes from 1 mL to 10 mL further significantly reduced microbial loads in anolyte-treated samples ($P<0.05$) following anaerobic incubation. As the FAC in anolyte decreases rapidly upon contact with organic material, this finding highlights the importance of supplying excess anolyte-treatment volumes. The improved performance observed with the larger ratio of anolyte solution to tissue is in keeping with the understanding that more activation energy and reactants will be available to act, suggesting that an irrigation stream may be the ideal mode to apply the solution. Increasing the strength of anolyte for further improved performance in treating DFUIs is also an option. In 2020 an EU Biocidal Products Committee published a risk assessment and opinion noting no cause for concern for topical skin use of anolyte at 300 ppm FAC [40]. Carefully designed randomized control trials will be necessary to further explore the efficacy of anolyte immersion on improved DFU healing outcomes.

Our study is slightly limited considering that 39/51 of the ulcers sampled were superficial and lacked infection symptoms. Diagnosis of DFUIs is often based on subjective identification of typical infection symptoms [24], which are less clearly defined in those with immune dysfunction, neuropathy or ischaemia. We found that only 4/42 (9.5%) DFUs which yielded $>10^5$ cfu/g tissue exhibited clinical infection symptoms indicating that DFUIs are very likely underdiagnosed. The association of *S. aureus* and higher anaerobic microbial loads (Supplementary Table S3) amongst patients with a prior LEA history is attributable to the presence of chronic and biofilm-infected DFUs that are more difficult to manage and therefore more likely to be LEA-associated. Anolyte has previously been shown to be non-toxic, microbiocidal and capable of penetrating biofilms [16,35], therefore further study specifically investigating its suitability for deep and chronic wound treatment is warranted.

Species identification was performed on isolates selected from each sample and a selection of treatment types and incubation conditions in order to carry out a limited survey of the range of organisms present. Interestingly, species such as *S. agalactiae*, *Finegoldia magna*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Prevotella buccalis* previously recovered from the oral cavity or gastrointestinal tract [41—44] were recovered from 17/51 (33.3%) DFU samples collected. Of the 12 patients who were sampled on more than one occasion, only one patient (P4) yielded an identical monomicrobial culture composed of *P. mirabilis* on two separate occasions (Supplementary Table S1). Distinct microbial populations were revealed on separate occasions from the other 11 individuals, indicating that DFU microbial populations are dynamic and fluctuate.

Unsurprisingly, *S. aureus* was the predominant species recovered (30/51 ulcer samples, 58.8%) correlating with previous investigations, however in contrast to these studies, no MRSA were detected [11,45]. The *S. aureus pvl* gene often associated with skin and soft tissue infections, was absent (Supplementary Table S4), correlating with previous studies of DFU-based *S. aureus* populations [46]. The low prevalence of antibiotic resistance genes reflected a relatively diverse MSSA population, likely correlating with the superficial nature and absence of biofilm of the wounds sampled. Sequence type replacement occurred in two patients (P4 and P19) over two years. In two patients (P17 and P18), multiple ST1 isolates recovered over four months had distinct *spa* types (P17; t002 and t067, P18; t067 and t1082), whereas persistent STs and *spa* types were identified in three patients (P20, P24, P29) over a four-month period (Supplementary Table S4). In each of these three patients, isolates from individual patients recovered on separate occasions were identified as the same ST, *spa* type and exhibited a maximum of four allelic differences (Figure 2). Strain type persistence amongst patients was complicated by the identification of two distinct ST5 clusters (Clusters 1 and 2; five isolates from five patients per cluster) and a ST1 cluster (Cluster 3; two isolates from two patients) of closely related isolates in each case. Isolates within clusters were very closely related (range 0—3 allelic differences) suggesting isolate transmission between patients (Figure 2). The possible release of fine particulate matter in clinical treatment areas following debridement procedures, or the congregation of patients with open wounds in clinical waiting areas may have led to microbial transmission between patients and warrants further investigation.

Routine DFUI treatment typically includes oral and intravenous antibiotic administration for superficial and invasive infections, respectively. These treatments can be problematic in patients who have ischaemia, vascular disease or kidney disease, all of which are very common diabetes-related complications. Our study revealed that 200 ppm anolyte immersion is highly effective for debrided DFU tissue disinfection ($P<0.0005$), resulting in an average 99% (2 log) microbial load reduction following 3-min immersions relative to saline-treated tissues. The reduction of average and median microbial loads of anolyte-treated tissues to $<10^5$ cfu/g indicates that the microbial burdens were reduced to levels that would not impede wound healing [9]. Furthermore, anolyte safety has been previously demonstrated using reconstituted human oral epithelial models [16].

In conclusion, regular anolyte-based DFU immersion has excellent potential to provide a non-toxic, inexpensive and highly effective means of DFUI prevention and treatment, in combination with conventional wound treatments such as debridement, dressing and offloading.

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Conflict of interest statement
The authors declare no conflicting interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2023.06.006.

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