

Original Research Paper

# The Role of Living, Resident Bacterial Biofilm Populations in the Osseointegration of Oral Implants

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**Abstract:** This study presents a human jawbone model which explains bone health, disease and quality and the osseointegration interphase, at the cell level. Curation of 454 pyrosequencing data obtained from 153 bone, serous-effusion and granulation samples taken from 40 patients during two-stage dental implant installation, revealed a 20 phyla jawbone microbiome with total genera of 387. Multivariate statistical ordination of curated pyrosequencing data revealed six distinct ecologic groups in healthy and pathogenic ecosystems. Pyrosequencing of pristine bone (found in sites of congenital tooth absence) revealed a resident, health biofilm population, which now serves as an ecological health control and is a critical health reference in longitudinal multivariate statistical ordination of live biofilm, in population-shift. Osseointegration of a sterile, inert, commercially pure titanium surface which bio-mimics the surface and microarchitecture of pathogen-evading, smooth, uniform, healthy human cells, may define a persistent, beneficial association with symbiotic competence, where cooperative colonisation proceeds between beneficial bacteria, host stem-cells and biocompatible biomaterials, without immunomodulation. Dampening or restraint of the host immune response by beneficial bacterial sRNA is now reported in animal models and may be a key element in symbiotic homeostasis. We confirm that the clinical effect of surgical debridement and perfusion of an encapsulated, pathologic biofilm nidus to a vascularised health margin, beyond the confining sclerosis, is microbial population-shift. Population-shift can only occur in living biofilm communities. Enduring osseointegration can only be achieved by ensuring ecologic recovery of the bone bed to homeostatic health, that is, a bone bed that supports diverse, stable, resistant, resilient and even biofilm communities, with consortial regeneration of both healthy osseous internal and cortical architecture, before implant installation. The authors recognise that further research and replication studies of both pristine human jawbone and other bone qualities are required to provide additional knowledge and verification of our findings.

**Keywords:** Resident Bacterial Biofilm, Biofilm-Based Osseointegration, Oral Implant, Regenerative Surgical Debridement, Population-Shift, Enduring Osseointegration, Immunomodulation

## Introduction

### *Cellular Homeostasis and Pathologic Disturbance in Human Jawbone*

This study used DNA molecular microbiology as 454 pyrosequencing with titanium reagents to curate

resident polymicrobial bacterial biofilm assemblages from 153 bone, serous effusion and granulation samples taken from 40 patients.

Our findings suggest that osseointegration occurs in the presence of resident, homeostatic biofilm populations and symbiosis research may now extend to human biomaterials with more complex multi-partner

associations, including biotic and abiotic surfaces (Hentschel, 2021). The resident populations may be subjected to ecologic disturbance from tooth-borne infection and therefore present variable bone qualities, defined in microbial ecology across a clinical gradient of health and disease. Non-metric Multidimensional Scaling (NMDS) ordinated the curation of the pyrosequencing data into six distinct ecologic groups according to biofilm phenotype (Nelson, 2015), consistent with contemporary categorization of health and disease (Ciofu *et al.*, 2022). In this way, health and disease may be defined at cell-level.

The Nelson and Viljoen bone quality index (Viljoen, 2019) describes five microbial ecologic categories across alternative stable ecologic states, which includes alternative stable pathologic ecologic states (Shade *et al.*, 2012). This index describes two health states (H2 = health, higher bone density; H3 = health, lower bone density) and three disease states (D1 = sclerotic bone density, D4 = lytic bone density and D5 = mixed sclerotic/lytic bone density), where the potential for the regeneration of internal osseous architecture is defined by the variation in the site-specific resident biofilm phenotype. Both sclerotic bone (D1) and lytic bone (D4) are diseased bone characterized by disruption of normal trabeculation (Parfitt, 1962). Normal histologic variation is defined by Schroeder (1991), where the trabeculation is connected and varies only in coarse or fine trabecular mesh patterns and intra-trabecular density. The cortical envelope is of normal thickness without sclerotic densification. The D1, D4 and D5 biofilm disease states need to be bio-remediated before implant installation by Regenerative Surgical Debridement (RSD), to recover ecologic health and normal internal osseous architecture (now defined as H2 or H3), where ecologic diversity and stability supports symbiotic colonization of health microbiota on abiotic and biotic surfaces at the osseointegration interphase, without immunomodulation.

With factors like a sterile surgical field, clinical surgical competence and no intraprocedural or manufacturing contamination of the implant surface, the outcome at the osseointegration interphase will be delivered by the ecologic health/disease status in the bone bed and the resident, live biofilm phenotype in the bone bed (Ciofu *et al.*, 2022). Furthermore, the surface topography and microarchitecture of the implant surface (Sa value, or surface roughness) should biomimic the osseous ground substance (Brånemark *et al.*, 1985). Percival *et al.* (2015) describes the smooth, uniform surface of healthy human cells as “pathogen-evading”, while Mukherjee and Bassler (2019) describes such (smooth) surfaces as “inhibiting cell-to-cell communication quorum sensing” by retaining microbial exposure to cell (or implant) surface micro-fluidity which dilutes and clears pheromone autoinducer cells, keeping

their numbers below a threshold level. If micro-fluidity is disrupted by a rough surface topography or microgrooves, this may reactivate pathologic persister-cell dormancy and phenotypic relapse infection (Lewis, 2010, Fisher *et al.*, 2017). The key issue is that the microbes of interest are 1-2 microns, so an interpillar surface separation of less than 1 micron ( $Sa < 1\mu$ ) will prevent bacteria slumping and adhesion onto surface recesses (Graham and Cady, 2014), thereby retaining exposure to cell surface micro-fluidity, with concomitant inhibition of cell-to-cell communication via quorum sensing (Mukherjee and Bassler, 2019).

In contrast to a sterile bone model, where the principal issue is the upregulation of pluripotent mesenchymal stem cells in response to implant installation (note that this model gives no consideration to competition from resident bacterial bone “guests”, in a “race for the implant surface”) (Gristina *et al.*, 1988), the ubiquitous biofilm bone model attaches great import to surface roughness, because of the increased adhesion of bacterial biomass (including pathogens). Where the microbes of interest are 1-2 microns (Graham and Cady, 2014), roughened surfaces with an interpillar distance of more than 1 micron significantly increased adhesion of bacterial biomass and pathogens (Bermejo *et al.*, 2019), with both increased surface area for adhesion (Berne *et al.*, 2018) and facilitated activation of quorum sensing and ultimately, endotoxin production. Costerton *et al.* (1978) stated that adhesion plays “the central role in the success of pathogenic bacteria. Prevention of adhesion is an effective way to combat or prevent bacterial infection”.

An abiotic implant surface that is rougher than the biotic surface may create preferential microbial adhesion with potential loss of genera that contribute to the resilient homeostasis of the resident biofilm (Ehrlich *et al.*, 2008). Thus, a hard-won population-shift, recovered to ecologic homeostasis, by what may have been repetitious surgical debridement, may be destabilized by genotypic response to the surface, with the loss of constituent genera, creating a potential impediment to symbiotic, cooperative, homeostatic colonization at the osseointegration interphase. Pathogenicity may re-emerge in populations that are missing normal constituents and ultimately, there is agreement in both theory and experimentation in macroecology that “the removal or addition of even a single species can cause drastic community changes”, leaving simplified, lower diversity communities, more susceptible to invasion and disease (McCann, 2000).

Costerton *et al.* (2003) suggested that “a resident biofilm population may preclude colonisation by pathogens”, maintaining cellular homeostasis by avoiding pathways of pathologic capillary lysis, which includes direct endotoxin lysis from gram negative pathogens (Brånemark and Urbasschek, 1967) and compression lysis from inflammation and raised intraosseous pressure (Wannfors and Hammarström, 1989;

Wannfors and Gazelius, 1991). Cellular health and normalized function rely on normalized resident microbiota (Van Oppen and Blackall, 2019). Lasting ecologic diversity in beneficial resident microbial symbiosis underlies “mechanisms of health, resilience and cellular homeostasis” (Visick *et al.*, 2021).

Interestingly, Visick *et al.* (2021) state that we now recognize that in resident biofilm populations there are “a much greater number and greater complexity of beneficial microbes in our bodies”, compared to pathogens, where normal resident, beneficial, live biofilm communities sustain biologic health defined in ecologic and histologic cellular homeostasis (Van Oppen and Blackall, 2019). The superior numbers of beneficial bacteria, coupled with the smooth and uniform machined implant surface and its propensity to evade pathogen adhesion and thereby inhibit quorum sensing, provides the platform for the enduring success of osseointegration.

Supporting this is evidence from an animal model that some bacterial sRNA was able to dampen and restrain the immune response (Moriano-Gutierrez *et al.*, 2020) and hence reduce the inflammatory disturbance to a short-term pulse disturbance without transition to a less stable, lower diversity alternative ecological state (Shade *et al.*, 2012). This may play a key role in sustaining a symbiotic, cooperative colonization of diverse beneficial health microbiota on abiotic and biotic surfaces at the osseointegration interphase, progressing a passive, enduring, osteoblastic anchorage without an immunobiological event, immunomodulation or foreign-body response. This results in a lasting symbiosis between mammalian host cells and their supported, healthy microbiota (Visick *et al.*, 2021).

Weinstein and Darouiche (2001) observed that the ubiquitous nature of biofilm meant that we could circumvent pathologic biofilm to either less pathogenic or non-pathogenic biofilm, but not remove it. Costerton (2007) described a disturbance to a resident biofilm population which resulted in the complete or partial replacement of that population by a more disease orientated population as “population-shift”. Bioremediation and circumvention of pathologic residency before implant installation could be achieved by regenerative surgical debridement to a vascularized margin, through the sclerotic encapsulation. The clinical effect of the debridement was population-shift. The health ecology of a future implant bone site could be recovered with extinctions and additions of phyla, according to habitat fitness, via a microbial convergence of the reconnected, adjacent health microbiota (Bartow-McKenney *et al.*, 2018).

We hypothesize that enduring cellular homeostasis in jawbone supporting osseointegration requires a stable vascular nutrition, with a connected, healthy capillary microvasculature, a stem cell rich periosteum, supporting histologically stable microenvironments with

connected trabeculation which sustains beneficial, ecologically diverse, resident biofilm populations, which are both resistant and resilient to pathogen invasion. This is achieved by passive osteoblastic anchorage and symbiotic, cooperative colonization of health microbiota on inert abiotic and biotic surfaces, without immunomodulation. Biocompatibility requires biofilm compatibility.

As Botton *et al.* (2006) stated “It is time therefore to revisit the existing concepts and hypotheses and discuss their applicability to ecosystems ruled by microbial processes”.

## Materials and Methods

### Patient Selection

Forty patients requiring osseointegrated dental implant therapy were prospectively enrolled into the study as they presented. All surgical implant sites were two-stage, full-flap, sterile surgical procedures, ad modum Brånemark (Adell *et al.*, 1981). Ethical approval was obtained from the Human Research Ethics Committee, The University of Sydney (HREC reference number 07-2007/9962). Patients were issued with a patient information statement and gave written informed consent.

### Sampling

Multiple samples were taken from the same site to increase the probability of sequencing the entire ecological community. Samples were obtained from osteotomies in all four dental quadrants, within post-extraction regenerated or regenerating jawbone and were defined as:

- a) Inherited Apparently Healed Bone (IAHB) samples taken from an apparently healed edentulous site where extraction of the infected tooth had been done previously in another dental practice (n = 29). The extraction protocol had conformed to current teaching where the diseased tooth was extracted without socket intervention, with the expectation of spontaneous bony healing and preservation of bone sterility
- b) Debrided Apparently Healed Bone (DAHB) samples taken from an osteotomy prepared in regenerated bone which had apparently healed for at least 3-6 months after a surgical debridement of an infected tooth socket beyond sclerotic confinement or surgical debridement of osteolytic/osteosclerotic “apparently healed” edentulous bone to an ecological health margin (n = 76)
- c) Congenital Absence Bone (CAB) osteotomy samples were taken from two patients presenting disease-free bone sites with congenital absence of upper lateral incisor teeth (n = 9). These sites had never supported

a tooth and were thus deemed to contain disease-free bone. One site also had both deciduous and permanent congenital tooth absence

### Sample Collection

Blood and tissue fluid samples were taken by paper point effusion, using sterile size 80 Maillefer endodontic paper points. Two sterile paper points were held in place in the sample site for a minimum of 15 sec and transferred to vials containing Reduced Transport Fluid (RTF) with 30% glycerol and frozen. Where concern existed for dilution of the sample by a transport fluid medium, paper points were placed in dry sterile vials and frozen. Bone samples were retained on the drill flutes and placed in the vials and frozen.

Samples included:

- Serous effusion
- Granulation/fibroconnective soft tissue
- Bone retrieved from the flutes of round surgical guide drill
- Bone retrieved from sterile surgical bone filter
- Bone retrieved from infrabony lesions
- Bone retrieved by surgical excision using rongeurs

Samples were then transported to Macquarie University, Sydney, Australia, extracted and subjected to PCR amplification using the 27F 16s rRNA eubacterial universal primers. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Bacterial Tag-Encoded FLX Amplicon Pyrosequencing (bTEFAP) of the V1-V3 regions of the 16S rRNA gene was commercially performed by Molecular Research Laboratory USA using the Titanium platform (Roche). This bTEFAP method has been described by Dowd *et al.* (2008) and utilized in characterizing a wide range of environmental and health-related microbiomes.

### Scanning Electron Microscopy

During our initial research, SEM was carried out on multiple samples to confirm that we were dealing with living bacteria in biofilm communities and not planktonic bacteria, or dead bacterial remnants. This is discussed further in discussion.

The samples were fixed in 3% glutaraldehyde for 24 h, dehydrated through increasing concentrations of ethanol and immersed in hexamethyldisilane (Sigma-Aldrich) 50% for 10 min and 100% for 3×10 min, before being aspirated and evaporated dry overnight. They were mounted on metal stubs and sputter coated with 20 nm gold film. The samples were imaged in a JEOL 6480 LV scanning electron microscope (Japan electron optics

Laboratory, Japan) with a voltage of 10 kV and a viewing distance of 20 mm.

### Statistical Methods

Many variables and data sets with up to 94% zero cells were detected. As shown in results the need for non-parametric analysis of the groups dictated the use of:

- a) Univariate Shannon-Weiner index of alpha diversity together with the jack-knife standard error
- b) Multivariate analysis by ordination with Non-Metric Multidimensional Scaling (NMDS)
- c) Analysis of Similarities (ANOSIM) was used to test the significance of a difference between groups. Similarity Percentage (SIMPER) was used to define the compositional differences within and between the groups

Non-constrained non-parametric multidimensional scaling analysis in 3D were presented for all ordinations. Many runs of ordination were performed with bray curtis similarity measure and the Sørensen Incidence-based Index to achieve the method with the least stress value. This method of NMDS analyses the matrix of pair-wise similarities between samples in the data set. Similarity or dissimilarity can be measured. Analysis of similarities using bray-curtis similarity index was chosen to do this as it uses quantitative data and not just presence or absence of information. If assigned groups such as IAHB or DAHB are meaningful, samples within groups should be more similar in composition than samples from different groups (pisces conservation).

An ANOSIM significance test ranks similarity within and between groups and compares this with similarity generated by random chance. A square root transformation is chosen to normalise non-parametric data.

### Contamination

Contamination of bone samples containing exogenous microbes has been problematic in previous endodontic studies (Siqueira and Lopes, 2001). Therefore, to detect for contamination, at the time of bone sampling, additional samples were taken from saliva and plaque and the microbial assemblages compared between sites using Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis of the 16S rRNA gene. A 918bp gene fragment of 16S ribosomal RNA was amplified using bacterial universal primer 6-FAM fluorescent dye labelled primer 8F (5'-Fam-AGAGTTTGATCCTGGCTCAG-3') and 926R (5'-CCGTCAATTCMTTGTGAGTTT-3'). The 25 µL reaction mix contained 1X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 200 µM dNTP, 400 nM forward and reverse primer, 1U Immolase Polymerase (Biolone) and 100 ng DNA template and was subjected to 95°C for 10 min,

denaturation at 95°C for 15 sec, annealing at 56°C for 45 sec and extension at 72°C for 2 min and a final extension step of 72°C for 10 min. The PCR products were purified using QIA quick PCR purification kit (Qiagen) and digested with 20U restriction enzyme HhaI (New England Biolab) at 37°C for 3 h. The fluorescent labelled terminal restriction fragments were separated by capillary electrophoresis using ABI 3130 genetic analyser and TRFLP profiles were analysed by comparison of abundance peaks.

## Results

### *Curation of Pyrosequencing Results*

Using DNA (culture-independent) analysis, all 153 clinical samples, including the two CAB samples, were positive for the presence of bacterial biofilm. The 20 phyla found in human jawbone and the number of samples with bacteria in each phylum is shown in Table 1. All implants were installed as two-stage procedures.

Surgical debridement protocols were developed over time (DAHB) meaning that some osteotomies preceded the introduction of this protocol or presented as non-debrided Apparently Healed Bone sites (IAHB).

To provide contemporary DNA-based verification of the sampling integrity, bone samples were accompanied by samples of the potential contaminants of plaque and saliva, taken at the time of the surgery for analysis of each of the three samples by TRFLP. This analysis is based on variation in the 16S rRNA DNA-fingerprinting technique to reveal community abundance peaks. In every sampling site, site-samples had different microbial assemblages and it could be concluded that each of the three samples were uncontaminated by the other.

Examples of TRFLP graphs, generated from samples taken at the time of osteotomy preparation and sampling and including saliva and plaque, are shown in Figs. 4b-d.

### *NMDS of Curated Pyrosequencing*

NMDS analysis provided multivariate ordination (piscis conservation) of the curated 454 pyrosequencing of all clinical samples, in 3D. NMDS places the most ecologically similar samples closest together and the least similar further apart with the best arrangement in a small number of dimensions determined by a parameter termed the “stress”. The best ordination will be the one with the lowest stress. Several ordinations were tried and compared with different starting positions, as recommended (Henderson and Seaby, 2014). Multivariate analysis is highly site-specific as it detects the effect of disturbances on biodiversity. NMDS is the multivariate

approach most suited to data in disturbance studies (Magurran and McGill, 2011).

Communities can be tracked through disturbance and the recovery trajectory, separated by ecological similarity or dissimilarity against CAB ecological health control. NMDS with the bray curtis (piscis conservation) similarity measure, revealed a clear separation of six ecologically distinct groups in the sample data with a good representation in reduced dimensions (3D stress = 0.17) (Fig. 1).

A total of 387 genera were identified across all samples. Surgically Debrided groups (DAHB) pooled microbiome results contain all of the 51 genera found in the health control (CAB) (Fig. 2), although across a clinical gradient of health and disease it was clear that some debrided sites achieved only partial ecological health recovery (DAHB Group 1) and required further intervention (i.e., more than one debridement) to create a ‘cleaner’ ecological recovery closer in ordination to the health control before implant installation. Non-debrided ordinated groups (IAHB) had significantly different genera in their compositional structure to the health controls. These groups ordinated closer to disease and biomaterial stability in such bone beds may be challenged by lower diversity and ambiguous ecological stability. The soft bone category is identified by NMDS analysis as a high pathogen subgroup, within non-debrided IAHB (Fig. 1).

### *Analysis of Similarity (ANOSIM). Statistical Significance Between CLINICAL Groups*

The results of an ANOSIM between the six ecologically distinct groups are shown in Table 2.

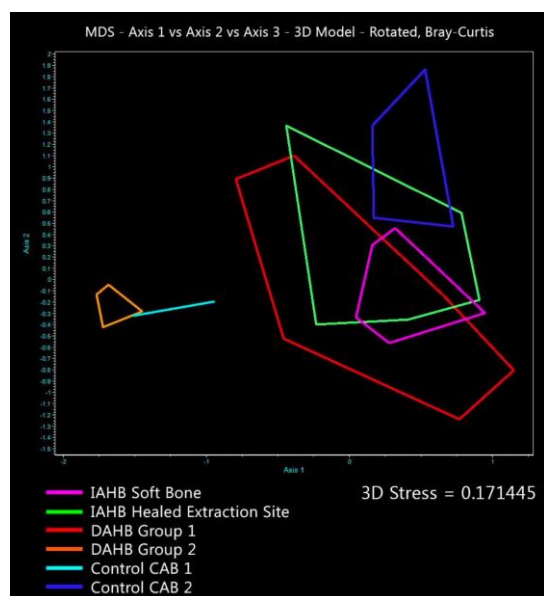
All pairwise comparisons were statistically significant ( $p \leq 0.05$ ) except for IAHB healed extraction site verses IAHB soft bone ( $p = 0.996$ ). An ANOSIM significance test, ranks similarity within and between groups and compares this with similarity generated by random chance. A square root transformation is chosen to normalise non-parametric data. ANOSIM tests whether the effects of disturbances on group compositional community structure are statistically significant (Magurran and McGill, 2011). Despite the ecological closeness of IAHB groups revealed in the NMDS ordination, a Similarity Percentages analysis (SIMPER) of the sample groups revealed significantly higher levels of known pathogens present in the IAHB soft bone group than in the IAHB Healed extraction site group (IAHB soft bone 89% versus IAHB healed extraction sites 75%). The osteolytic presentation of IAHB soft bone may be explained by its microbiological compositional structure. No osteosclerotic or soft osteolytic bone qualities were reported in DAHB samples.

**Table 1:** Overall Phyla: The human jawbone microbiome in health and disease. 20 phyla present in 153 clinical samples from extraction sockets, Inherited Apparently Healed Bone (IAHB) (n = 29), Debrided Apparently Healed Bone (DAHB) (n = 76) and Congenital Absence Bone (CAB) (n = 9)

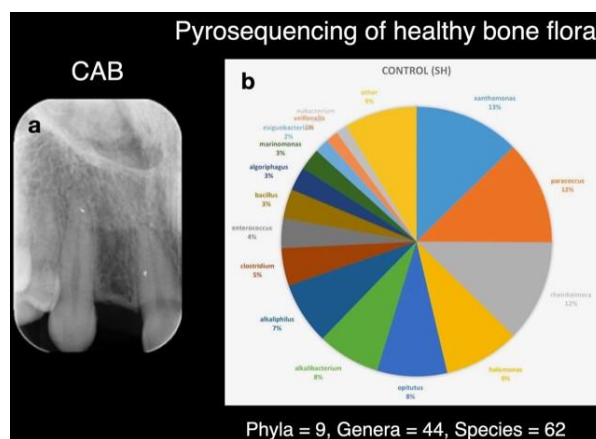
Phyla	No. of samples	Phyla	No. of samples
Firmicutes	135	Gn02 (candidate division)	12
Actinobacteria	134	Verrucomicrobia	12
Proteobacteria	133	Chloroflexi	10
Bacteroidetes	115	Chordata	7
Fusobacteria	75	Planctomycetes	6
Tm7 (candidate division)	49	Thermi	5
Cyanobacteria	37	Nc10 (candidate div)	2
Spirochaetes	24	Spam (candidate div)	2
Tenericutes	21	Acidobacteria	1
Synergistetes	17	Aquificae	1

**Table 2:** Analysis of Similarity (ANOSIM): The results of an Analysis of Similarity (ANOSIM) are shown. All pairwise comparisons were statistically significant ( $p \leq 0.05$ ) except for IAHB (healed extraction site) versus IAHB (soft bone), where ( $P = 0.996$ )

	DAHB group 1	DAHB group 2	IAHB (healed extraction)	IAHB (soft bone)	Control CAB1	Control CAB2
DAHB group 1	-	0.001	0.001	0.050	0.003	0.001
DAHB group 2	0.001	-	0.001	0.001	0.048	0.001
IAHB (healed extraction)	0.001	0.001	-	0.996*	0.005	0.003
IAHB (soft bone)	0.050	0.001	0.996*	-	0.018	0.001
Control CAB1	0.003	0.048	0.005	0.018	-	0.028
Control CAB2	0.001	0.001	0.003	0.001	0.028	-



**Fig. 1:** Example of an initial non-constrained Non-Parametric Multidimensional scaling analysis in 2D (NMDS), with the bray-curtis similarity measure, revealed the possibility of six ecologically distinct groups in the sample data set. A clear cluster of significantly different groups was evident (3D stress = 0.17)

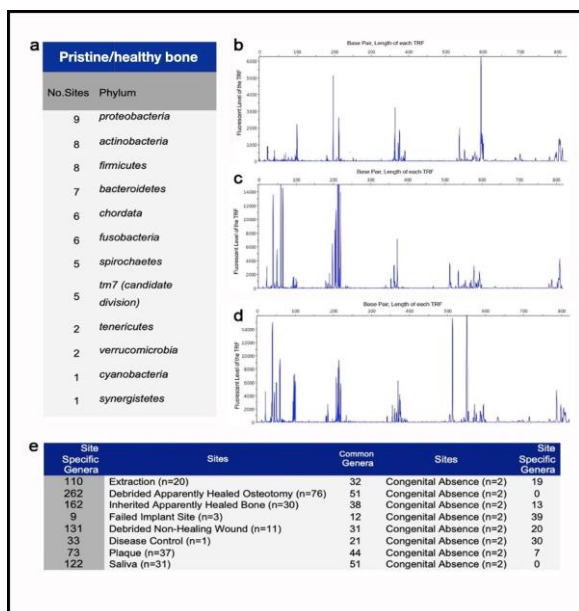


**Fig. 2a-b:** (a) A site of congenital tooth absence. This site also had congenital deciduous tooth absence; (b) The results of CAB curation contradict the presumed sterility of human jawbone and make the case for a resident 'health' microbiome. The undisturbed state of this site includes a living, permanent, resident, homeostatic biofilm population. Microbial diversity high in richness and evenness. Only 8 of 44 genera more than 5% abundance and only 11 of 44 genera more than 1%





**Fig. 3:** Control CAB2 with 6-year case review of both upper lateral incisors



**Fig. 4a-e:** (a) curation of taxa at the phyla level for bone samples from pristine sites of congenital tooth absence. 12 total phyla with pyrosequencing sample integrity confirmed by community abundance profiles using TRFLP; (b) TRFLP plaque as potential contaminant. X axis = base pair, the length of each Terminal Restriction Fragment (TRF). The Y axis = fluorescent level of the TRF; (c) TRFLP saliva as potential contaminant; (d) TRFLP Congenitally Absent Bone (CAB) as health control. All three samples share sample integrity, none of the three samples are contaminated by the other; (e) microbial diversity in health (CAB) and disease. Pooled pyrosequencing comparison of genera between distinct ecological groups versus the 51 genera revealed in the CAB health control. Only sites subjected to surgical debridement and the saliva contamination control contained all 51 genera, which sustain homeostatic health. The category most ecologically dissimilar to homeostasis was the failed implant containing only 12 similar genera from 51

### Ecological Health Control

DNA molecular 454 pyrosequencing of bone samples from healthy sites of congenital tooth absence in the adult human jaw would appear to be without precedent (Fig. 2a).

All sample sites (n = 9) were taken from two healthy adults in disease-free mouths. Both were non-smokers with no compromising comorbidities and both were positive for the presence of biofilm communities in the osteotomy sites (Fig. 2b). One site in the position of the upper permanent lateral incisor had congenital absence of both deciduous and permanent teeth. The results of CAB curation contradict the presumed sterility of human jawbone bone and is considered evidence that even healthy jawbone harbours a (beneficial) resident biofilm population. These populations define a homeostatic health control reference for jawbone as an ecological health control (Fig. 2b) in microbial compositional structure at the level of phyla and genera.

The identification of resident biofilm communities in sites of congenital adult tooth absence represent control sample groups, to be compared with continued NMDS ordination of disturbed communities, in order to monitor their recovery and health status prior to implant installation. In this way, it may be possible to develop surgical debridement methodologies adapted from orthopaedic experience with chronic osteomyelitis. This ultimately may be a benchmark for multivariate analysis to identify alternative stable states that may favour improved clinical outcomes in jawbone osseointegration.

The outcome of two implants in CAB2 bone (healthy/pristine) is seen in Fig. 3 after 6 years of function.

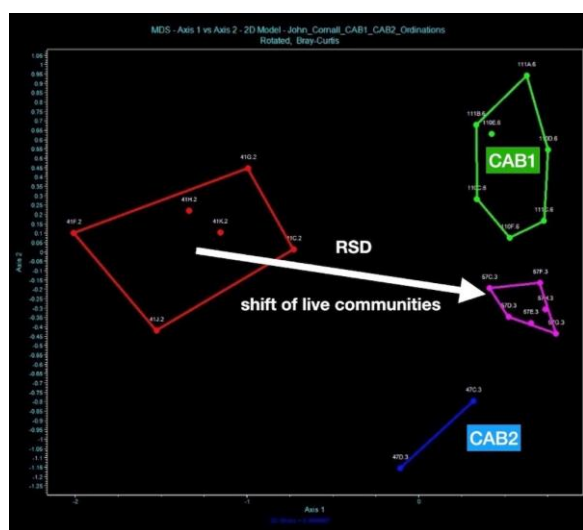
Variable bone quality could now be defined as a microbial health/disease phenomenon across a spectrum of non-pathologic to pathologic microbial community assemblages. Pooled results of ecological similarity revealed that only surgically debrided sites and saliva control sites had all 51 genera of the health control (Fig. 4e). Site specific bone quality reflected site-specific dominance of beneficial health or pathogens in microbial community structures (Kolenbrander, 2011). Absence of trabeculation meant that regeneration had been impaired and osteosclerosis was associated with all chronic bone pathology (Parfitt, 1962). The category most ecologically dissimilar to homeostasis was the failed implant (Fig. 4e).

The primary contribution of bone bed dynamics to implant outcomes is to restore health ecology, which may now be referenced against the ecological health control of pristine bone of Congenital tooth Absence (CAB).

Dental implant failure as a dual biofilm infection of both the implant surface and chronic osteomyelitic infection of bone (Zimmerli, 2014) achieved the most significant ecological pathogenicity with 39 genera, distinct from the 51 genera of the health-control, even more significant in pathogenicity than the chronic periapical granuloma (disease control), with 30 distinct genera, which had previously been described as a sterile inflammatory process.



**Fig. 5:** Results of an NMDS ordination. Congenitally absent tooth sites from two patients separated into two microbially distinct groups, Control CAB1 and Control CAB2



**Fig. 6:** NMDS multivariate statistical ordination of curated pyrosequencing before and after repetitive RSD. Biofilm population-shift referenced against CAB1 and CAB2, verifying them as unique health-controls and confirming that the resident biofilms are living. The red area represents persistent, chronic pathology at 9 months post-extraction and the turquoise area represents the recovered health microbiome through population-shift, following multiple debridement's. Sample taken at 12 months post-extraction and ordinated against both CAB1 (green) and CAB2 (blue) health-controls. Note the separation of microbial health and disease through a population-shift to a healthy microbiome, plotting distant to the persistent disease sample and now plotting close to health controls CAB1 and CAB2

### Comparison of the Exponential H (Alpha Diversity), CAB Against IAHB and DAHB

Constituent pooled phyla, genera, species or Operational Taxonomic Units (OTUs) were examined using Shannon Wiener exponential H alpha-diversity (Pisces Conservation) for richness and evenness in various clinical states and sample types. Exponential H Shannon Wiener diversity for distinct clinical groups, shows the CAB health-controls with the highest pooled alpha diversity (exponential H). There is a maximised diversity disparity between the failed implant and the diversity of the ecological health-control; the former is 3.9 times lower in diversity. Botton *et al.* (2006) noted that ecologic diversity and stability are almost inseparable ecological concepts.

A comparison of the alpha diversity was also made between the CAB control and both DAHB and IAHB. DAHB (partially or fully ecologically recovered bone) was more diverse than IAHB (Table 3).

### Congenital Absence Bone (CAB), Dissection into Two Groups

The initial model of congenital absence bone was dissected into 2 groups, control CAB1 and control CAB2. Ordinal separation was confirmed by a significant difference in microbial assemblage composition (Fig. 5). This may imply that even in pristine/healthy bone, the microbial community assemblages may be unique for each patient.

Both members of CAB groups were designated as controls, since the microbial assemblages of each were situated in clinically pristine, non-diseased bone.

### NMDS Validation of Health Controls

NMDS ordination allows us to visualise population-shift in the bone bed following RSD and also validates the health controls CAB1 and CAB2 as valid reference points of bone-bed microbial health. If RSD resulted in a population-shift away from our two health controls, then they may not be representative of bone health/homeostasis (Fig. 6).

### Visualization of Longitudinal Population-shift in Same-Site Osteotomy Following Implant Failure in Soft Bone (D4), Followed by RSD and Successful Revision

#### Using Pie-Charts

Figure 7 shows three pie charts constructed from pyrosequencing of same-site constituent genera (simpler analysis) in Sample 54F (soft bone-D4), sample 56E (implant failure-D4) and sample 91E (post-debridement health recovery-H3), which correlates with improved bone quality and recovery of ecologic diversity, richness and



evenness in genera, as determined from longitudinal univariate exponential H alpha diversity statistical analysis.

#### Using Exponential H Alpha Tabulation

Analysis of a longitudinal series of samples, taken at the initial implant placement (54F), then later at the time of implant failure (56E) and then again after RSD (91E) is shown in Table 4. The initial bone site supported 6 phyla and 18 genera (alpha H 7.9) and alpha H dropped to 1.147 at the time of implant failure with just 3 phyla and 9 genera. After successful RSD, the bone bed was found to support 11 phyla and 53 genera, with an alpha H of 27.8. The revision implant successfully Osseo integrated in the ecologically recovered bone bed.

#### Using Shannon-Wiener Exponential Diversity Analysis

Recovery of ecological diversity within the implant bone bed can be shown using Shannon-Wiener exponential diversity analysis (Fig. 8).

#### Example of the Clinical Effect of RSD on Population Shift in Debrided (Gp2) Versus Inherited, Non-Debrided Bone

NMDS maps live biofilm extinctions and additions according to habitat fitness, revealing the clinical effect

of surgical debridement as population-shift (Fig. 9). Multivariate analysis is highly site specific and therefore highly relevant to mapping disturbed communities through the recovery trajectory, over time (Warwick and Clarke, 1993).

It considers the identity of species, rather than richness or diversity (Hewitt *et al.*, 2005). NMDS may be the most appropriate multivariate approach for data retrieved from disturbance studies (Magurran and McGill, 2011).

#### Example of Longitudinal Same-Site Population-Shift Before Implant Installation

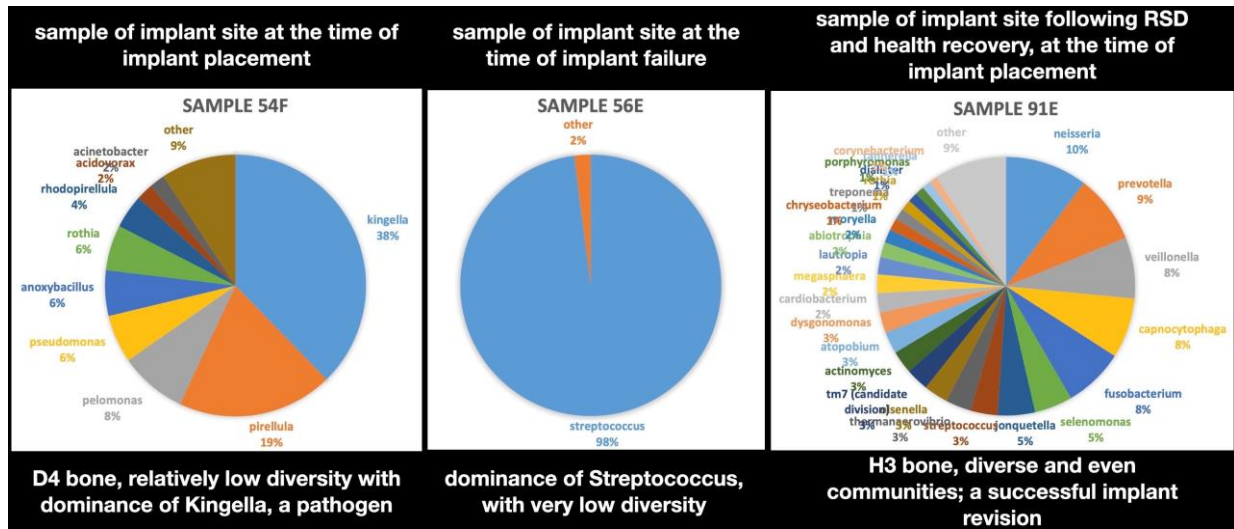
Immediate implant placement (that is, at the time of tooth removal) has become an accepted protocol, however, the validity of this protocol may be questionable, as our NMDS ordination exposes the risk of this protocol in placing the implant into a diseased bone bed. Figure 10 shows ecologic recovery of a potential implant site before implant installation. The patient presented with a fractured post-core and crown and therefore the site was deemed unsuitable for immediate implant placement. The bone-bed was diagnosed as D5 (osteolytic/sclerotic) and was debrided at the time of tooth removal, in preparation of implant placement.

**Table 3:** Alpha diversity (exponential H) of CAB versus other samples; CAB health-control as an ecological diversity/stability reference exponential H. Note CAB has the highest alpha diversity, nearly 4 times that of a failed implant

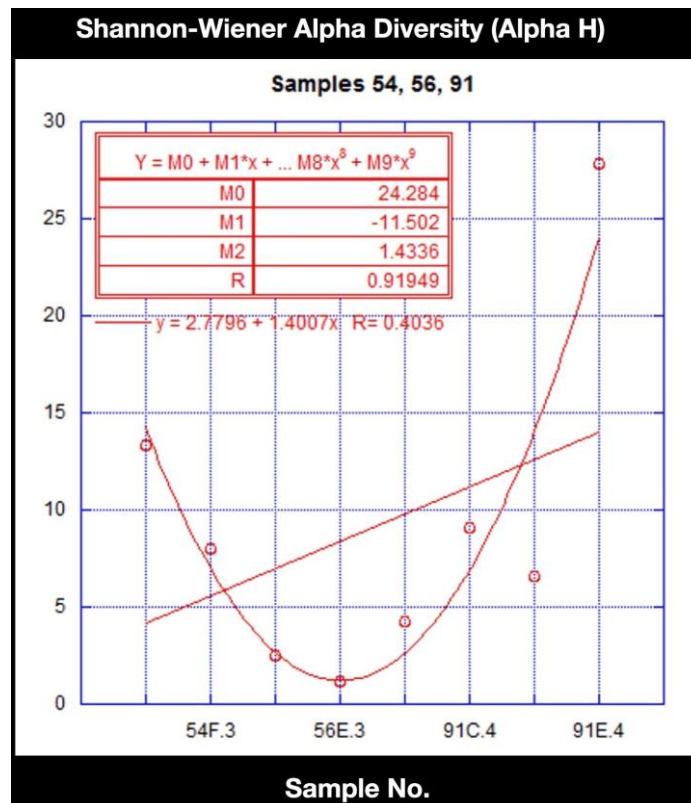
	No. of patients	No. of samples	No. of phyla	No. of genus	No. of species	Alpha diversity (Exp. H)
Failed implant site	1	3	5	21	42	2.630
Extraction socket	8	16	14	142	296	7.125
Saliva	23	31	15	173	397	7.315
IAHB	13	30	15	200	423	8.549
Plaque	26	37	14	117	303	8.907
DAHB	21	76	17	313	714	9.108
CAB	1	2	10	51	73	10.346

**Table 4:** Analysis of exponential H Alpha diversity of samples 54F, 56E and 91E; longitudinal same site univariate exponential H alpha diversity and microbiological analysis of the above three same site samples, showing alpha H diversity, phyla, genera and a debrided population-shift and bone quality recovery from D4 bone to H3 (Viljoen, 2019) bone with significant increase in ecologic diversity in health

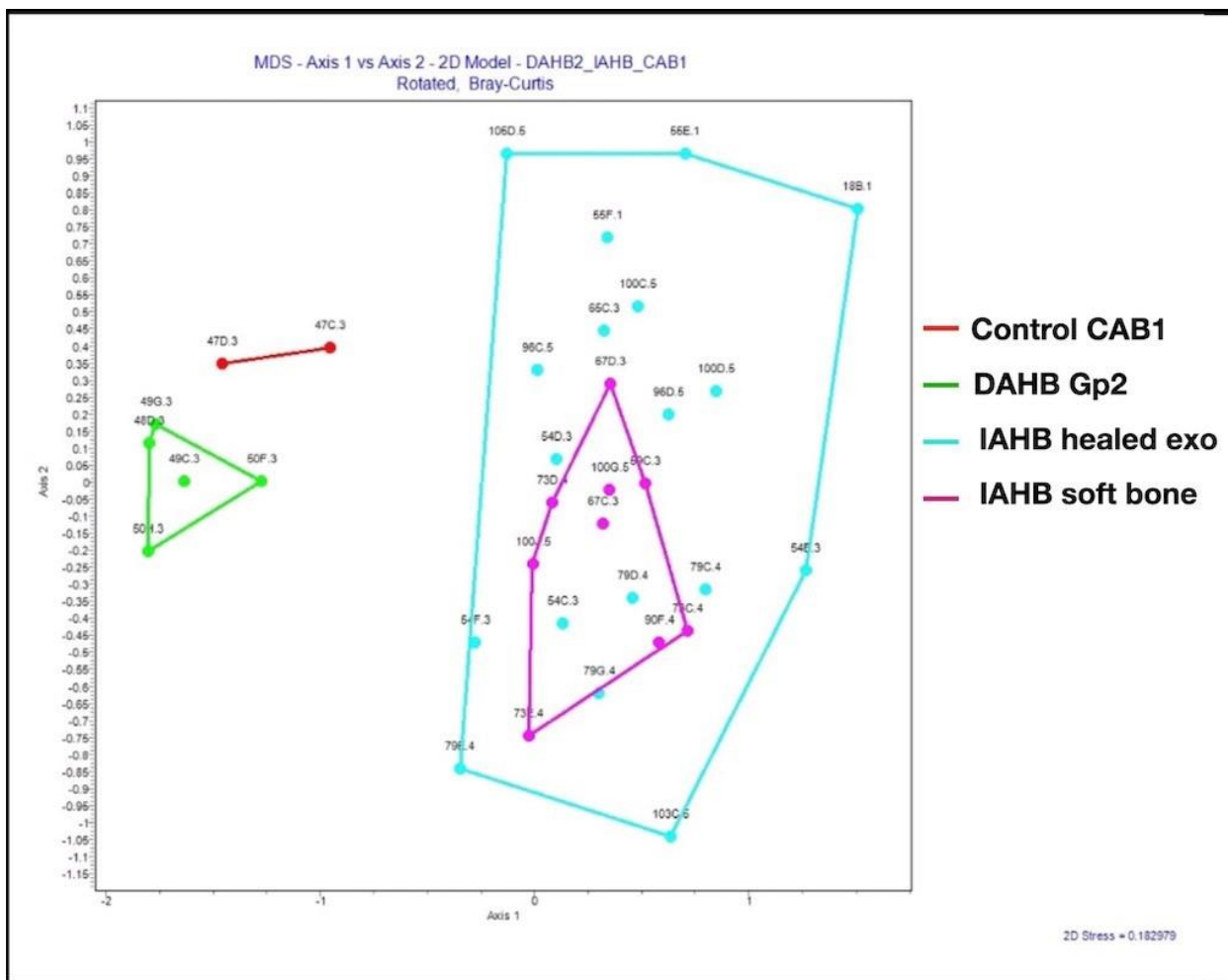
	Sample at initial placement	Sample at the time of implant failure	Sample post-RSD
Sample No.	54F	56E	91E
Phyla	6	3	11
Genera	18	9	53
Bone quality	D4	D4	H3
Alpha H	7.970	1.147	27.822
<i>Streptococcus</i>	1.6%	97%	3.4%
Diversity	Not very	Very low	rich



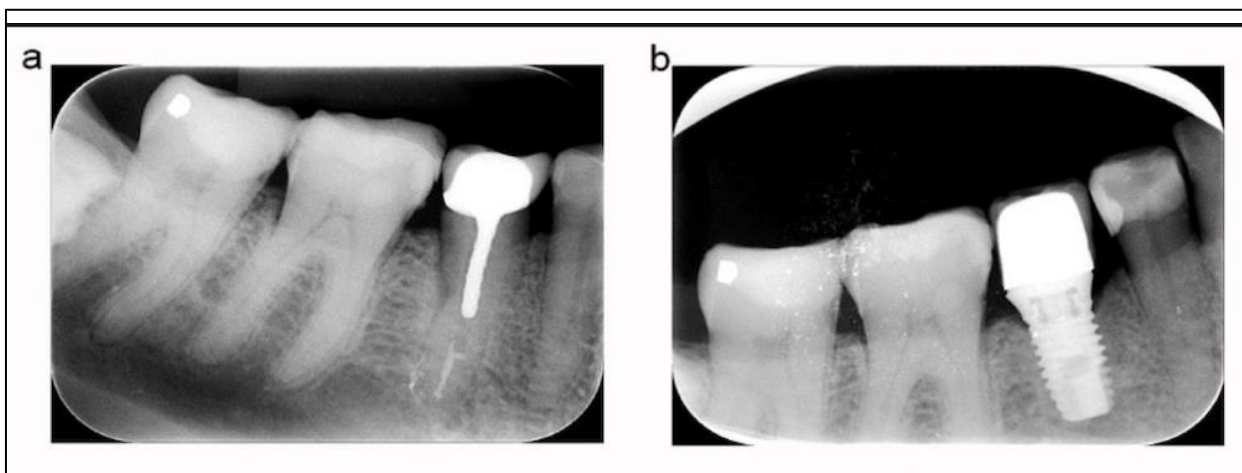
**Fig. 7:** Longitudinal pie charts constructed from pyrosequencing of constituent genera in a same-site implant osteotomy: 54F-initial osteotomy in poor quality, osteoporotic, soft, non-debrided D4 bone at first implant installation. Note relatively low diversity. 56E-short term infective implant failure. Note very low diversity, close to single genus biofilm pathology. 91E-following RSD there is a return to ecologic diversity and evenness, confirmed by univariate exponential H statistical analysis. This results in improved bone quality with a return to normal histologic bony trabecular and cortical architecture (H3). Successful same-site revision followed the eradication of biofilm disease

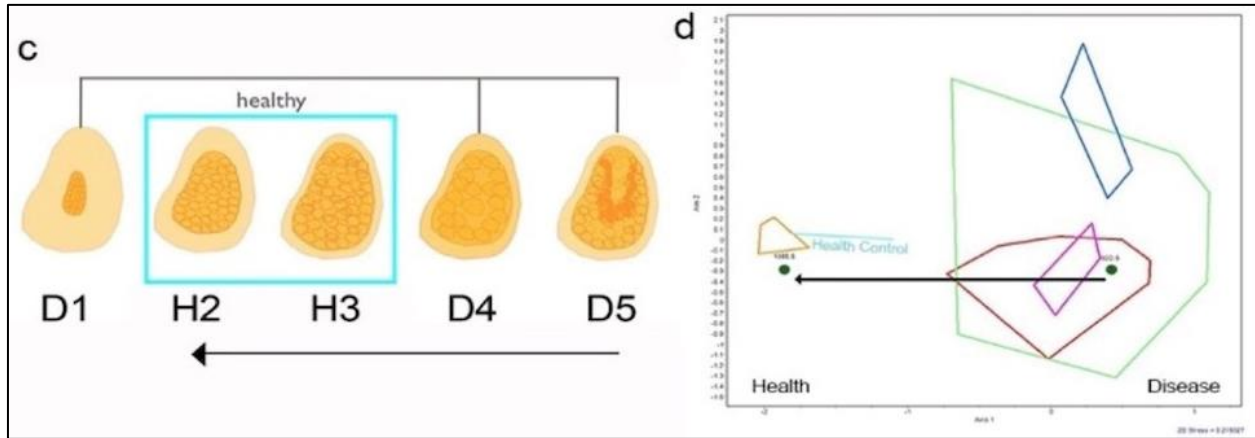


**Fig. 8:** Univariate Shannon wiener exponential H diversity analysis showing recovery of ecologic diversity and bone quality in revision osteotomy 91E: D4-H3 (Viljoen, 2019) following regenerative surgical debridement after primary failure in soft “osteoporotic” D4 bone. Implant failure 56E records lowest exponential H and 12 species of *Streptococcus* in what is effectively a single genus biofilm pathology

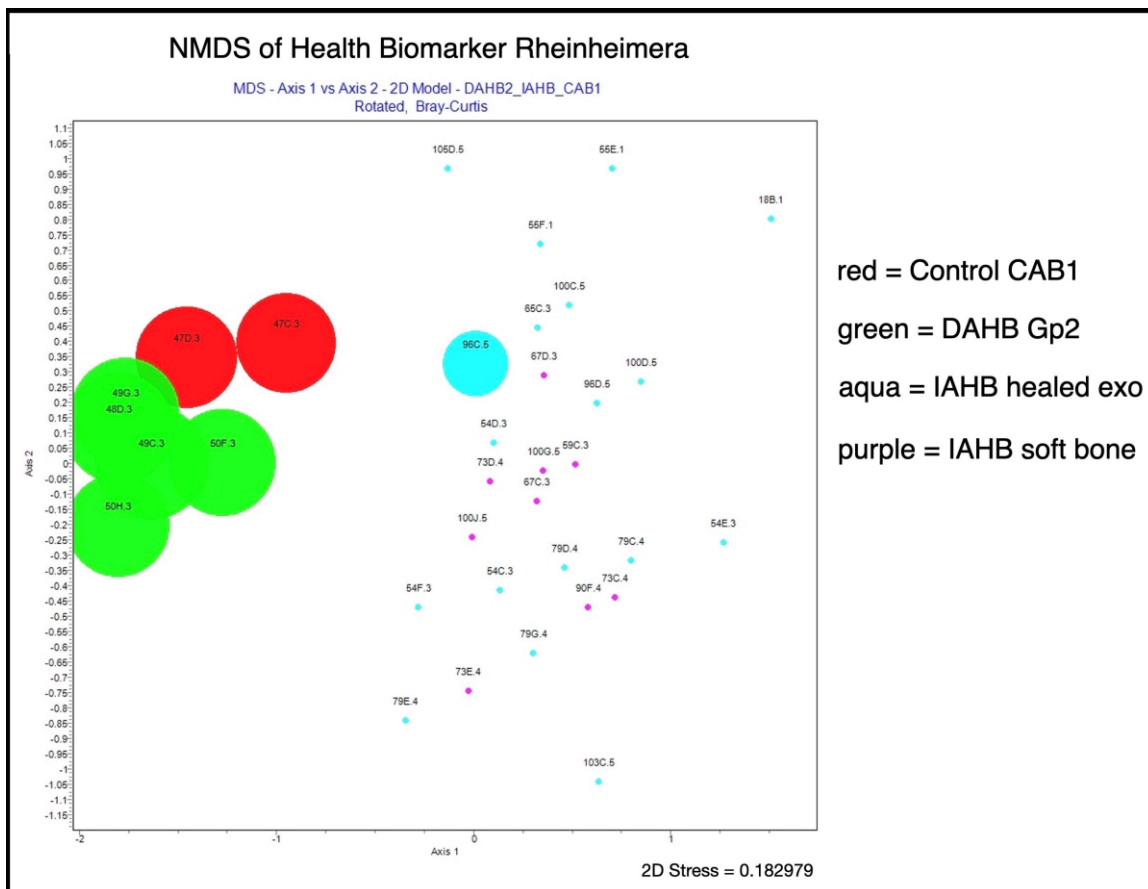


**Fig. 9:** NMDS ordination of soft, osteolytic bone plots as a high pathogen subgroup of IAHB (non-debrided bone), ecologically distant from health control. DAHB 2 plots close to the CAB health control, while IAHB healed extraction and IAHB soft bone subgroups plot distant to both CAB and DAHB Gp2

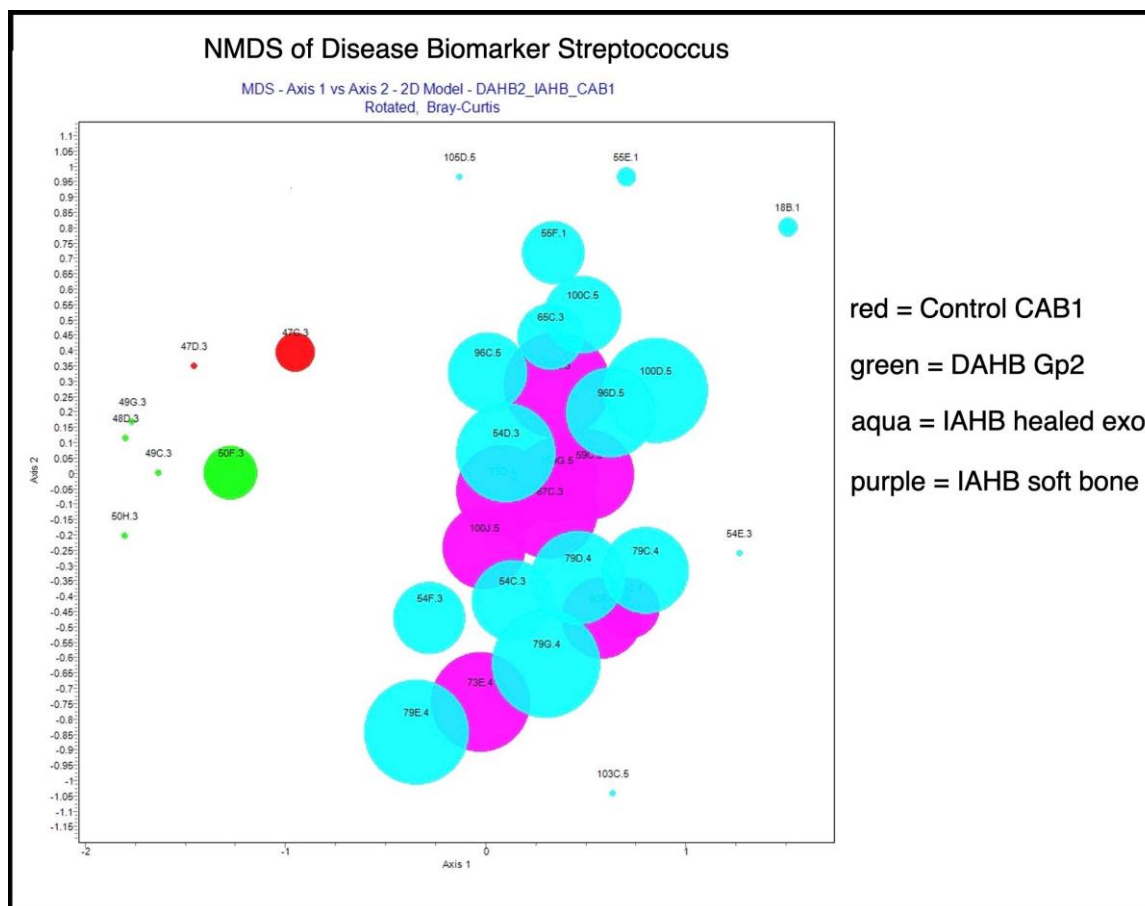




**Fig. 10 a-d:** Improved ecological bone health by population-shift, recovers diversity and bone quality; (a) Extraction and surgical debridement of D5 mixed lytic/sclerotic pathologic bone lesion with vertical root fracture of lower right second premolar; (b) Regenerative surgical debridement beyond sclerotic zone of D5 bone bed delivers population-shift to H2 bone quality. This bone-bed supports a successful implant; (c) As a result of RSD and a population shift, the bone bed returned to normal, healthy H2 architecture; (d) NMDS ordination of curated pyrosequencing at extraction, then post-debridement at implant installation. Note complete population-shift from pathologic to non-pathologic resident biofilm through a single RSD, with a dominance of the health biomarker genus *Rheinheimera*. However, patients should be warned that multiple debridements may be required



**Fig. 11:** NMDS single genus ordination of *Rheinheimera*, a health biomarker



**Fig. 12:** NMDS single genus ordination of *Streptococcus*. *Streptococcus* is a pathogen biomarker

High pyrosequencing abundance of *Streptococcus* (often with cohort NMDS disease biomarker *Propionibacterium*), characterise resistant pathology, with significantly altered osteolytic/ostesclerotic D1, D4 and D5 (Viljoen, 2019) anatomy, correlating with pre-existing pathology of long duration and lower diversity. When both genera were curated in high abundance from the same site, *Streptococcus* also presented typically with high “species” (Operational Taxonomic Units) numbers and the sites were surgically difficult to recover ecologically, often requiring multiple debridements, especially in the posterior mandible and sites in the anterior maxilla with diffuse “bicortical” sclerosis.

#### NMDS of Health Biomarker *Rheinheimera*

In the curation of natural and pathogenic ecosystems, the genus *Rheinheimera* (Fig. 11) proved to be a predictive health biomarker in constituent genera, while *Streptococcus* (Fig. 12) and *Propionibacterium* proved to be predictive pathologic biomarkers, when in significant abundance in the community profile. NMDS ordination of the health biomarker *Rheinheimera* was undertaken of four bone groups including control CAB1, DAHB Gp2, IAHB (healed extraction site) and IAHB (soft bone). Note

how DAHB Gp2 ordinated closer to the control CAB1 than the two non-debrided groups of IAHB (Fig. 11).

#### NMDS of Disease Biomarker *Streptococcus*

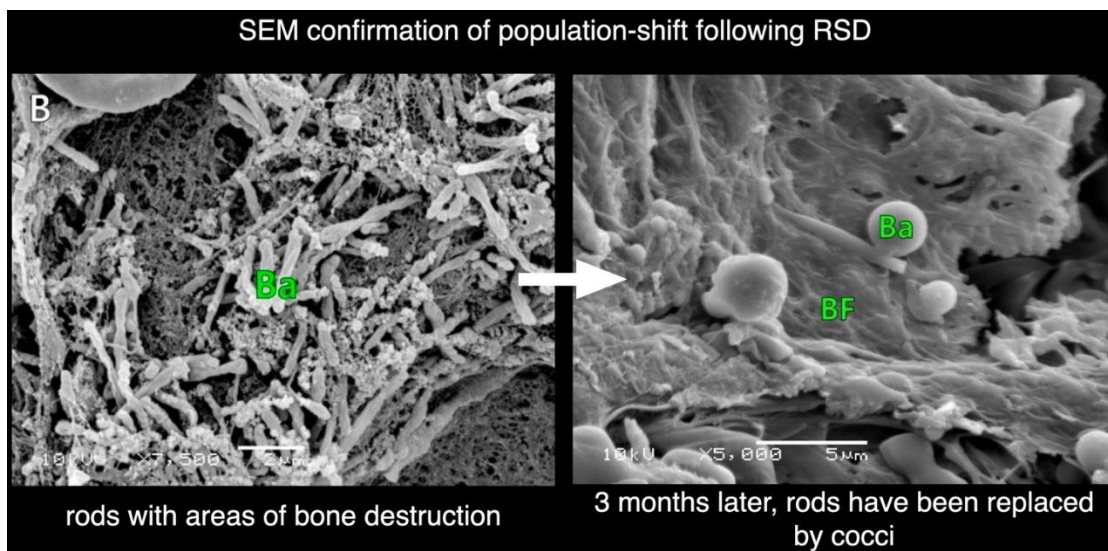
NMDS ordination of the disease biomarker *Streptococcus* was undertaken of four bone groups including control CAB1, DAHB Gp2, IAHB (healed extraction site) and IAHB (soft bone). Note how the two non-debrided groups of IAHB ordinate closer to each other and away from the health control CAB1 and the ecologically recovered DAHB Gp2 (Fig. 12).

### Discussion

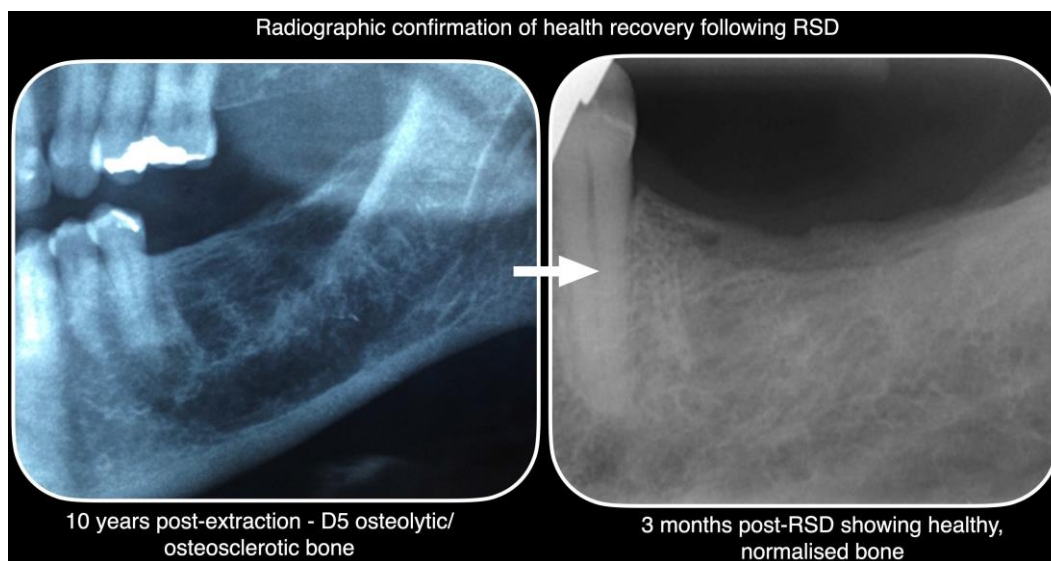
#### *Does Human Jawbone Support Living Bacterial Biofilm Communities?*

454 pyrosequencing is a method suited to the detection of bacteria living in biofilm communities, whereas culture techniques will only detect bacteria living as “free-floaters” or planktons (Clarridge III, 2004, Dowd *et al.*, 2008; Wolcott *et al.*, 2010; Tuttle *et al.*, 2011; Ehrlich and Arciola, 2012; Rhoads *et al.*, 2012; Tipton *et al.*, 2017).





**Fig. 13:** SEM image of a bone sample taken at 9 months post-extraction, with pathological, sessile, invasive rod-bacteria spreading over the bone surface with patchy areas of biofilm ( $\times 7500$ ). Following RSD at 9 months, at 12 months a population-shift to coccoid Bacteria (Ba) is noted, indicating that the bone-bed supports living microbial Biofilm (BF) populations (Nelson *et al.*, 2023)



**Fig. 14:** An example of radiographic return to normalized, healthy bone, following RSD

Site-specific pyrosequencing revealed an almost infinite number of alternative ecological states, in both healthy and pathogenic ecosystems (Shade *et al.*, 2012; Weinstein and Darouiche, 2001). This strongly suggests the routine presence of bacterial biofilm populations in the jawbone. 454 pyrosequencing does not distinguish between the DNA of living bacteria and dead bacteria and therefore, it could be argued that the presence of bacterial DNA in human jawbone is an end-product of the actions of the innate immune system. We have however, shown convincingly that, using orthopaedic-derived debridement techniques which link healthy tissue with niduses of diseased tissue, we can cause

a bacterial population-shift, back to a health population, which ordines close to our health controls. Only living bacterial biofilm communities can be population-shifted. Since RSDs can result in a population-shift that ordines closer to our health controls, this suggests that our health controls too are living microbiome communities (Fig. 6).

Figure 13 provides visual (SEM) confirmation of a longitudinal population shift from rods to cocci, following debridement and 3 months of healing. Figure 14 is an example of radiographic, post-RSD healing at 3 months, following 10 years of persistent osteolytic/osteosclerotic (D5) bone histopathology.



## Curation

Curation of our data found polymicrobial biofilm assemblages in 100% of jawbone samples. In all sites in the dental implant treatment sequence, starting with the extraction socket and including granuloma and congenital absence, we found polymicrobial biofilm assemblages, both in health and disease. To reduce bias in pyrosequencing sampling, multiple serous effusion, granulation and bone samples were retrieved from each site. One of the principal variations was to sample at different depths and at different widths within the jawbone. The pyrosequencing may suggest the existence of an infinite number of alternative stable states as micro-niches in both healthy and pathogenic ecosystems. The results indicate that considerable site specificity exists for each biofilm assemblage.

## Ordination

Ordination is a collective term for multivariate techniques which summarize a multidimensional dataset in such a way that when it is projected onto a low dimensional space, any intrinsic pattern the data may possess becomes apparent upon visual inspection (Pielou, 1984). Multi-variate analysis of the pyrosequencing results of our identified clinical groups reveals a progressive reduction in known pathogens as the sites reduce their ecological distance, in ordination, relative to the health controls.

It is apparent from the NMDS ordination that surgical debridement produces a wound with reduced levels of pathogens and is ecologically closer to the compositional structure of health microbiota in CAB1 and CAB2, with increased community diversity (Fig. 6). It is suggested that the ecological distance between DAHB Group 1 and DAHB Group 2 results from the fact that the two debrided groups represent alternative stable states, but in different phases of ecological recovery. The percentage of known pathogens retained in the compositional structure of pooled results of DAHB Gp1 may suggest that a repeated debridement would result in an ecological outcome which would ordinate closer to DAHB Gp2. In the transitional gradient between health and disease DAHB Gp2 is less pathogenic versus DAHB Gp1 and its ordination reflects this in its closeness to control CAB1.

Of the ecological distinct groupings in this study, the failed infected implant had lowest alpha diversity (Table 3) (Shannon Wiener exponential H) and the most genera distinct from the health control. Dental implant failure as a dual biofilm infection of implant surface accompanied by chronic osteomyelitic biofilm infection of bone (Zimmerli, 2014) achieved the most significant ecological pathogenicity, with 39 genera distinct from the 51 genera of the health control. Osteomyelitis is defined when an

infective microbe establishes chronicity. This is characterised by low grade sessile bacterial persistence and bony necrosis (Lew and Waldvogel, 2004).

Failure to eliminate even the smallest areas of necrotic bone areas may maintain infection for years (Ochsner, 2006). The presence of a residual and persistent sclerotically confined biofilm nidus in apparently healed bone is often in subclinical form that is highly recalcitrant and may persist for life (Percival *et al.*, 2011).

Ciorny (2011) describes a transition from a “dirty wound” to a “clean wound”. Weinstein and Darouiche (2001) describes a similar transition of the wound as progression from pathogenic to less pathogenic and non-pathogenic. Shade *et al.* (2012) describes each of the states including those with high relative abundance of known pathogens as “alternative stable ecological states”, but those closer to health are closer to their pre-disturbance homeostasis. It is suggested that these states are more diverse, less refractory with increased resistance and resilience to further disturbance (Zimmerli *et al.*, 2004).

This study presents strong evidence that the human jawbone biological space is never microbially inert and benefits from the presence of a resident microbiota whose ecological resistance and resilience defends the space from disturbance, that is, pathological colonisation and chronic infection (Costerton *et al.*, 2003). Beneficial bacteria are in far greater numbers compared to pathogens (Visick *et al.*, 2021) and may play a role in resilient recovery of cellular homeostasis by using sRNA to dampen the immune inflammatory state (Moriano-Gutierrez *et al.*, 2020). Stable biofilm communities may play the dominant role in developing normal habitat physiology and they will provide the dominant numbers of beneficial planktonic bacterial cells that engage in the “race for the surface” (Gristina *et al.*, 1988) with pluripotent stem cells for the implant surface. Those bacteria found to be “normal microbiota” contributing to host tissue health in a resident symbiotic homeostasis may create a lasting, persistent, beneficial association (Visick *et al.*, 2021), essential for an enduring osseointegration. Ecological diversity provides stability in the face of disturbance events and spatiotemporal change within the biofilm, minimizing the probability of extinctions (Botton *et al.*, 2006, Kolenbrander, 2011).

## Limitations and Future Directions

The authors acknowledge that further research is required to corroborate our findings and to further examine both CAB and other bone qualities for their resident microbial ecology. One method of RSD is described in this study; however, other methods may be as or more effective in recovering jawbone to microbial ecological health.

## Conclusion

Within the limitations of this study, the constancy of bacterial presence in jawbone samples, independent of health or disease status or healing time after disturbance, suggests the presence of a resident bacterial biofilm population in bone currently presumed to be sterile. That these communities can be population-shifted by RSD suggests that these are living communities. The pyrosequencing curation and multivariate ordination of samples from healthy sites of adult congenital tooth absence CAB1 and CAB2 provides an ecological health control in microbial compositional structure at the level of phyla and genera. The ecologically distinct clinical groups ordinated by NMDS varied across a clinical gradient of health and disease. NMDS ordination of longitudinal pyrosequencing of disease and recovered health ecology, referenced by the health control, demonstrated the constancy of resident live microbial biofilm communities by population-shift. This overcomes the inability of DNA molecular sequencing to differentiate live or deceased microbial populations. Recovered health ecology was not a return to sterility. Site-specific bone quality was a direct outcome of live microbial biofilm in health and disease.

Just as the two concepts of resilience (recovery/rebound of pre-disturbance condition) and stability are, in an ecological sense, inseparable, increased ecological diversity in constituent richness and evenness (currently at the level of phyla and genera) correlates directly with improved bone quality. This re-establishes the resident biofilm homeostasis by population-shift and becomes a core treatment protocol in biofilm-based osseointegration. Symbiosis with ecologic diversity will endure over ones with lower, less stable diversity, which is why microbial compositional shifts, recovering health in ecologic diversity and internal histologic osseous architecture, must occur before implant installation.

Further research is needed into how the resident bacterial biofilm populations influence bone quality, implant success and the underlying mechanisms behind enduring osseointegration.

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## Author's Contributions

**Stephen Nelson, Honghua Hu, Graham Thomas and Karen Vickery:** Conception and design, acquisition

of data, analysis and interpretation of data. Drafted the critical review for significant intellectual content. Final approval was given for submission.

**Anita Jacombs:** Study design, data collection and analysis. A critical review of drafted and intellectual content. Final approval was given for submission.

**Anand Deva:** Conception and design analysis and interpretation of data. Final approval was given for submission.

**Andre John Viljoen:** Acquisition of data relating to peri-implant bone loss, analysis and interpretation of data, drafted, edited and critical reviewed for significant intellectual content. Final approval was given for submission.

## Ethics

The study was conducted in accordance with the declaration of Helsinki and approved by the University of Sydney Human Research Ethics Committee (HREC) (reference 07-2007/9962).

## Informed Consent Statement

Written information about the study was provided to all participants and written, informed consent obtained.

## Data Availability Statement

Data are available from corresponding author on request.

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