SCANNING ELECTRON MICROSCOPY ANALYSIS OF BIOFILM ON DENTAL IMPLANTS EXPLANTED DUE TO PERI-IMPLANTITIS

by

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submitted by Jae Wook Chang in partial fulfillment of the requirements for

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in Craniofacial Science

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Abstract

Background:

About 20% of subjects receiving implants develop peri-implantitis (PI) that associates with progressive inflammation and bone loss around implants, often leading to implant failure. PI is caused by bacteria that accumulate in peri-implant space but the consensus on microbial profile is still lacking. Microbial sampling of PI lesions has largely focused on analyzing bacterial species that have been shed from implant surface and captured in the pocket fluid. The purpose of the present study was to investigate the morphotypes of bacteria in microbial ecosystem that covers the implant threads and explore whether different brands of implants favor different morphotypes and whether certain morphotypes were associated with more advanced disease.

Methods:

The implants (N=14) that were determined to have failed by the clinician were removed and instantly processed for scanning electron microscope analysis. The implants were imaged at three equally divided levels of the exposed area due to diseased bone loss. Bacterial morphotypes [cocci, rods, filaments, spirilla/spirochetes] in each level were further analyzed at higher magnification to enable identification and quantification by three examiners. The different types of surfaces, mobility and years in functions were correlated to the presence of specific morphotypes.

Results:

Implants removed due to PI demonstrated the presence of variable bacterial morphotypes that did not correlate to disease progression in our preliminary study. Some implants were dominated by filaments and others showed the presence of combinations of cocci and rods or mixed morphotypes

of spirilles/spirochetes. Rods and filaments were dominant species throughout the surfaces and cocci showed increased presence towards the apical third compared to coronal and middle thirds. There were significant differences in the morphotypes in the implants with TiUnite and SLA surfaces (except for cocci), with mobility and with more than 10 years of function.

Conclusions:

The profiles of morphotypes in biofilms of different implants with similar clinical presentation of PI were highly variable and did not clearly associate with implant brand. While there were significant differences between implants, interestingly, similar morphotypes on individual implants were found throughout the entire implant surface.

Lay Summary

This research examined the various forms and shapes of microbes and their biofilms in the failed dental implants from the patients that had been in function for years. The aim was to see if there were any differences in different morphotypes in comparison to the ones typical to periodontitis and investigate if there were any patterns of the dominant morphotypes in various locations of the biofilm. Furthermore, three additional variables were tested including implant surface characteristics, bone loss (mobility) and years of function. Once imaged with 5000-times magnification using SEM, the morphotypes were quantified under 4 different morphotypes (cocci, rods, filaments, and spirilla/spirochetes). High variability of morphotypes of bacteria on implants with similar clinical presentation were evident. Morphotypes tended to be similar throughout the implant surface although different between individual implants. The data suggest that PI can be caused by various types of biofilms composed of different microbiomes.

Preface

This research was completed and written by the author under the supervision of Dr. Hannu Larjava. Ethical approval was obtained prior to the commencement of this research (Protocol number H17-02478). Acquiring the scanning electron microscopic images was assisted by Dr. Gethin Owen. The assessment of images and quantification of the morphotyped were performed by the author and Dr. Hannu Larjava and Dr. Ya Shen. During the data collection stage of this investigation, the preliminary stage of this study was accepted for the poster presentation at the annual meeting of the American Academy of Periodontology in Novemeber 2020. The meeting was supposed to take place in Hawaii, USA, but due to COVID-19 Pandemic, the meeting was transformed into a full virtual platform. Therefore, the Poster and PowerPoint presentation was recorded and presented. Additionally, the same was also selected to be presented during the 'Research Day' at the Faculty of Dentistry, University of British Columbia, Vancouver, Canada on January 26, 2021.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

3-D: three dimensional

16srRNA: 16 Svedberg ribosomal ribonucleic acid

A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans

ANOVA: analysis of variance

C1q: the complement componenet 1q

COX: cyclooxygenase

DNA: deoxyribonucleic acid

ECM: extracellular matrix

E. coli: *Escherichia coli*

E. corrodens: *Eikenella corodens*

EDS: energy dispersive spectrometer

ELAM-1: Endothelial leukocyte adhesion molecule-1

EPS: extracellular polymeric substances

FISH: Fluorescent In Situ Hybridization

GCF: gingival crevicular fluid

HIF-1a: hypoxia-inducible factor

ICAM-1: intercellular adhesion molecule-1

IL: interleukin

iNOS: inducible nitric oxide synthase

ITI: International Team of Implantology

kV: kilovoltage

LPS: lipopolysaccharides

M: mole

mm: millimeter

MMP: metalloproteinase

MRSA: methicillin-resistant *Staphylococcus aureus*

N: newton

nm: nanometer

OPG: osteoprotegerin

PCR: polymerase chain reaction

pH: power of hydroge

PISF: peri-implant crevicular fluid

PGE_2: prostaglandin E2

P. gingivalis: Porphyromonas gingivalis

PI: Peri-Implantitis

PIPES: piperazine-N, N′-bis (2-ethanesulfonic acid)

PMN: polymorphonuclear leukocyte

PRF: Platelet-Rich-Fibrin

PSD: polymicrobial synergy and dysbiosis

R_a: Roughness average

RANKL: Receptor activator of NF-kappa b ligand

RNA: ribonucleic acid

rRNA: ribosomal ribonucleic acid

RUNX2: Runt-related transcription factor 2

S. aureus: *Staphylococcus aureus*

SEM: scanning electron microscope

S. intermedius: *Staphylococcus intermedius*

SLA: Sandblast, Large-grit, Acid-etched

S. mitis: Streptococcus mitis

sp.: species

spp.: species pluralis

T. forsythia: Tannerella forsythia

TIMP: tissue inhibitor of MMPs

TLR: Toll-Like-Receptor

TNF-α : Tissue necrosis factor – alpha

T. Socranskii: Treponema socranskii

μm: micrometer

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My love Juhee and our dear sons, Lukas and Mark.

I am grateful forever and I cannot describe how much I thank you all for unbelievable amount of support and trust. I truly wish the three-years of journey that we all took together reminds us of precious value of dreams that comes together with challenges behind. Thank you all for believing in me and this incredible journey was never possible without you.

Dedication

For Lukas, Mark and Juhee

Chapter 1: Introduction

Peri-implantitis is a biofilm initiated infectious condition that cause bone loss around dental implant that can progress quickly and lead to implant failure. About 20% of indivifuals receiving dental implants develop peri-implantitis that has many fratures similar to periodontitis (periodontal disease) including inflammation and bone loss. In the context of periodontal disease, the current understanding of the role of biofilm points to the “keystone” pathogens and dysbiosis. According to this consept, a spefic pathogen (or pathogens) drive the development of the biofilm to favor more pathogenic overprotective commensal bacteria in a special environment and in interaction with host. While the peri-implant and peri-implantitis microbiomes remain to be further established, it is generally believed to be similar to that of periodontitis but have less diversity due to special environmental niche. Specific bacteria present in peri-implantitis lesions have been studied by extracting the biofilm from peri-implant pockets and subjected to molecular biological analysis, often by 16s RNA sequencing. However, less attention has been paid to the biofilm analysis in its native form on failing implants. In fact, there are no comprehensive studies demonstrating the biofilm composition on extracted dental implants. The aim of the present study was to describe using high resolution scanning electron microscope the distribution of bacterial morphotypes on the failed implants and evaluate the association of different morphotypes to implant topography and clinical findings.

Chapter 2: Review of literature

Biofilm is defined as the "syntrophic consortium of microorganisms in which cells stick to each other and often also to a surface." The components of biofilm include extracellular matrix (ECM) and extracellular polymeric substances (EPS). Both are functional and structural support of biofilm, facilitating cell adhesion, cell-to-cell communication, and differentiation. Nevertheless, extracellular polymeric substances are the secretions of microorganisms by which biofilm can establish their colony. Therefore, along with different types of microorganisms in biofilm, it is EPS that determines the biochemical nature of biofilm. EPS are commonly polysaccharides and proteins, but it includes other molecules like pieces of DNA, lipids, and other molecules. It is the actual component that forms the shape of biofilm, and its volume takes '50 to 90%' of a biofilm's total organic matter. Due to the nature of these exopolysaccharides, these molecules protect the microorganisms within the structure. The microorganisms within a biofilm are safer and given more opportunities to flourish compared to planktonic bacteria. Simply, the exopolysaccharides are their barriers to any attacks and, therefore, bacteria in a biofilm more resistant to any pharmaceutical treatments such as antibiotic therapy. Additionally, these protective matrices can provide nutrients by trapping them from the outside of the matrix. They also support cellular recognition, aggregation, and adhesions to be able to build up their communities in 3-D shapes.

Bacterial biofilms are found in all environments and are not all pathogenic and some used in commercial applications. The food industry uses biofilm-based molecules to induce a gelatinous texture to fermented milk products as these are digestible. The plants need exopolysaccharides to attach to those bacteria with nitrogen-fixation to their roots and soil particles as a symbiosis relationship[1].

2.1 **Oral Biofilm**

Bacterial biofilms are present throughout the digestive track including oral cavity. Biofilm studies in dentistry have been reported for a long time and have had multiple breakthroughs in their methodologies. In oral cavity, the biofilms are adherent to surfaces such as mucosa, gingiva, tongue, and teeth. In the periodontal environment, supragingival biofilm is often considered as more cariogenic and subgingival biofilm as more periodontopathogenic. The former causes the demineralization of enamel, causing caries and the latter to cause the inflammation of the soft tissue leading to alveolar bone loss. The bacteria in the oral cavity especially around the periodontium have been investigated in numerous studies that reported more than700 species in oral biofilm. Approximately 200 species may be present in one individual and up to 50 species can be identified in one site[2].

An individual bacterium in isolation behaves differently than one that belongs to the oral biofilm as a member of the community. Therefore, the proper way to study these microorganisms would be in native biofilm that is challenging to researchers who need to mimick the reality of oral biofilms not interfering with the polymicrobial equilibrium regardless of the state (health or disease). Culturing a single species of bacteria bears tremendous challenges dealing with various factors such as growing environmental factors such as temperature, pH, nutrients, and oxygen level (aerobic/anaerobic status). Isolating and culturing single species is important for understanding their potential virulence factors. However, biofilm infections are polymicrobial and, therefore, molecular biology techniques have evolved to identify and quantify all bacterial species in a given biofilm. Real-time PCR technology combined with 16S rRNA sequencing is one powerful way to analyze the composition of bacterial biofilm.

2.2 **Bacteria and biofilms associated with various forms of periodontal disease**

In a milestone study by Loe and Theilade (1965), it was shown that oral biofilms that accumulate on tooth surfaces after ceasing oral self care cause inflammation in the gingiva. Resuming the biofilm removal resulted in rapid recovery of gingival health[3]. Soon, the research community led by The Forsyth Institute (Socransky, Haffajee, Tanner), Royal College Copenhagen (Slots), State University of New York at Buffalo (Genco, Slots, Zambon) and University of Michigan (Loesche, Syed) initiated research programs to find "the specific pathogens" causing periodontal disease[3]. The pioneering papers by Listgarten (1976,1978) using dark-field microscopy revealed the main bacterial morphotypes associated with gingival health and disease[4]. Listgarten (1976) described disease severity in different categories (health, gingivitis, periodontitis, periodontosis, post-periodontosis) with specific morphotypes. He described the healthy flora with Gram-positive coccoid bacteria as a predominant type. Filamentous forms and Gram-negatives microorganisms were present but low in numbers in health. In gingivitis, coccoid and filamentous forms were present with a mixture of Gram-positive and Gram-negative species. The biofilm of gingivitis was thicker reaching 400 µm with abundance of intercellular matrix with fibrillar structures. Bacterial co-aggregations representing "Corncobs"-like structures (long filament covered with coccoid bacteria) were occasionally present with filamentous bacteria at the surface. Mobile bacteria such as spirochetes were also occasionally present. In periodontitis lesions, dense biofilm mass was present on the tooth surface similar to gingivitis but with the more prominent presence of various morphotypes including filaments and motile bacteria. Bacterial aggregations (cell-cell adhesions) were also more frequently observed in forms of "corncobs" and "with "bristle brush" formations. The central backbone of "bristle brushes" was one or a few large filamentous bacteria with Gram-negative rods or short filaments inserted on the top. Other species in

periodontitis included the various-sized spirochetes with Gram-negative bacteria with concave bodies and multiple flagella[4]. He reported that the morphotypes of microorganisms were relatively consistent regardless of individual variations between teeth and subjects. The formation of 'bristle brushes' and flagellated and motile forms were unique morphotypes in periodontal pockets.

With evolving analytical techniques, the polymicrobial complex was introduced and investigsted[5]. Socransky et al. (1998) used more than 13,000 plaque samples using 'whole genomic DNA probes and checkerboard DNA-DNA hybridization. Utilizing the 16S rRNA technique over the conventional culturing methods, Socransky et al. were able to establish the different microbial complexes associated with periodontal health and disease. Socransky and Haffajee (1998) performed the cluster analysis on more than 13,000 subgingival plaque samples from 185 adults.[6] The authors divided the pathogens into early colonizers and late colonizers using color schemes. Yellow, green, blue and purple complexes were the early colonizers and orange, and red complexes were considerd as the late colonizers. In this study, *Aggregatibacter actinomycetemcomitans* was categorized independently. Red complex bacteria were *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*. The special features shared by these pathogens along with *A. actinomycemetmcomitans* were the ability to penetrate into gingival epithelium (invasion)[7] and transform the sites into the hyper-inflammatory state.[6] Virulence factors of each pathogen are slightly different. However, the fundamental natures of virulence are somewhat similar in context of attachment, invasion, colonization and local tissue destruction resulting in the modification of host response[8].

P. gingivalis is one of the most investigated periodontal pathogens and is an obligate anaerobic, Gram-negative coccobacillus with no motility. O'Brien-simpson et al. (2004)

highlighted the importance of virulence factors of *P. gingivalis* with fimbriae, reinforced chemotaxis, fibrinolysin, degradation of immunoglobulin, hemolysin and gingipain. *P. gingivalis* possesses lipopolysaccharide (LPS) that is crucial antigen to trigger the host immune response in developing periodontal diseases. LPS is one of the most common yet critical virulence factors of *P. gingivalis.* LPS is a lipid endodoxin of bacteria with core oligosaccharide and O-specific polysaccharide. It is O-specific polysaccharide that is biological active and pathogenic[9]. IgG_2 subclass is the main antibody recognizing them. Upon exposures of LPS, human gingival fibroblasts secrete the cytokines such as IL-1b, IL-6 and TNF-a to increase the state of inflammation[10].

Fimbriae are filamentous structure to enhace the attachment of *P. gingivalis* onto the surface of host cells, extracellular matrix, or other bacteria. There are several types of fimbriae including the major FimA and minor Mfa that control the baceterial dependence of molecules and substances resulting in the formation of biofilm. Phenotypically, the major fimbriae FimA has length of approximately 3 μm and the minor short fimbriae are about 60-500 nm[11]. They play an important role in anchoring, binding and facilitating the adhesion to the host cells and to other pathogens as co-adhesion.[9]

Majority (85%) of proteolytic activity of *P. gingivalis* is performed by gingipains. Gingipain was named from '*P. gingivalis* clostripain', which belongs to 'cysteine' protease family present in the outer membranes as either in vesicle form or extraceullar structures of *P. gingivalis*[12]. Main activities of gingipains include increase of osteoclastic activity, dyregulate complement system (specially at C3, C4 and C5), dysregulation of polymorphonuclear cells and increase of vascular permeability resulting in higher recruitment of leukocytes[13]. These are divided into two groups: arginine-dependent gingipain R (Rgp) and lysine-dependent gingipain K(Kgp). Rgp has a

subgroup of RgpA and RgpB based on its chemical structure. Common features of their chemical structures include N-terminal domain, C-terminal domain, immunoglobulin superfamily-like domain (IgSF), signaling peptide, hematogglutinin/adhesion domain (HA) and catalytic domain (CD)[14]. Ito et al. (2010) reported that gingipains can also contribute to biofilm formation by co-aggregating *P. gingivalis* with other species such as *T. denticola* in dental plaque[15]. This is one of the examples of synergistic relationship expressing collective virulence of biofilm found in dental plaque among red complex microorganisms. Protelytic activity of gingipains include various types of extracellular matrix in the host tissue degrading to minimize the damage by host immune system. However, the main pathogenic activity of gingipains is to resist all three complement system pathways. All these three pathways have different initiating molecules, but the end points are same by creating the membrane-attack complex. *P. gingivalis* effectively decompose C5 convertase by activating pro-thrombin to thrombin[16]. *P. gingivalis* is one of the most common bacteria found in the various periodontal diseases including chronic periodontitis, aggressive periodontitis and necrotizing periodontitis. [17]

Tannerella forsythia is an anerobic, gram-negative fusiform bacterium with virulence factors such as lipopolysaccharides, extraceullar proteolytic enzymes and antigenic surface proteins on the outer membrane[13]. Relatively little is known about this patogens due to its difficulty culturing. Investigations have revelaed that chemical structure of LPS of *P. gingivalis* and *T. forsythia* were similar with S-from containing long multiple ladder-shaped step[18]. Proteolytic enzymes are produced by cell surface proteolytic enzymes including trypsin-like serine proteases[19]. Its unique virulence factor is characterized by its symbiotic relationship with *Streptococcus sanguis* and its minimal virulent pathogens not being able to degrade host proteases inhibitors[13].

Investigation using PCR, it was evident that *T. forsythia* was evident 91% and 9% of subgingival plaque samples from chronic periodontitis patients and healthy patients, respectively[20]. Another virulence factor of *T. forsythia* is one of the surface protein in the outer membrane inducing apoptosis of lymphocytes by forming pores of lymphocytes[13].

Treponema denticola is one of the red complex pathogens that has motility as one of its virulence factors[13]. Peri-plasmic flagellum is antigenic and facilitate invasion with motility. However, those are embedded inside between the outer sheath and cytoplasmic membrane. Also it has lipopolysaccharide inducing pro-inflammatory cytokines and major sheath proteins causing apoptosis and chemotaxis[21]. Dashper et al. (2011) explained that *T. denticola* may co-exist with *P. gingivalis*, but the alveolar bone loss was more prominent when *T. denticola* had more dominance in chronic periodontititis[21] The authors highlighted more virulence factors such as 'outer sheath vesicles'(OSV) containing proteolytic enzymes, adhesins and toxins with highly regulated mechanism. These vesicles may have their significance to express its presence in competition environment. This particular pathogen is dominant species in the nectrotizing gingivitis/periodontitis and healthy sites do not harbor these pathogens. Dentilisin is known to be *T. denticola's* major virulence factor and it is located as one of the surface proteins bound by outer membrane. Dentilisin degrades extracellular matrix and interferes host immune signaling[21]. Trypsin-like protease activity was also noted as one of the virulence factors of *T. denticola* and its pathogenic activity is more intense than that of *P. gingivalis*.

Aggregatibacter actinomycetemcomitans is a capnophilic, facultative anaerobic coccobacillus. Fives-Taylor et al. (1999) highlighted the various virulence factors of *A.*

actinomycetemcomitans[22] including leukotoxin that cause apoptosis of immune cells by forming a hole on the leukocytes. Similar to others, *A. actinomycetemcomitans* shares similar virulence factors such as invasion to resist phagocytosis, LPS to induce pro-inflammtory cytokines, fimbriae for adhesion to host cells, Fc-binding proteins to block the binding of antibody to the pathogen, cytolethal distending toxin (CDT) to supress the immune system and bacteriocins which is lethal to other bacteria for competition for nutrients[22]. There are six serotypes (a-f) and serotype b is known to be more prevalent in aggressive periodontitis [13]. In murin periodontitis study, Ebersole et al. (1995) reported that serotype b was the most virulent next to the serotype a followed by the serotype c[23].

The types of pathogens of chronic periodontitis are highly hetegrogeneous with these four forementioned bacteria; *P. gingivalis, T. forsythia*, *T. denticola* and *A. actinomycetemcomitans.* In addition, the orange complex organisms were common as well in residing with red complex organisms[6]. The microbiota of aggressive periodontitis was dominated with gram-negative anaerobic rods with capnophilicity. Specially in localized aggressive periodontitis, it was known to be *A. actinomycetemcomitans* that were a predominant species with upto 90% of prevalence[24]. Along with red complex, there were more prevalence of orange complex as well such as *Capnocytophaga, Eiknella corrodens, Prevotella intermedia, Campylobacter rectus*. Even though it was omitted from the current classification, refractory periodontitis was shown to have more orange complex organisms such as *Fusobacterium nucleatum, Prevotella intermedia, Parviromonas micra* along with *T. denticola* rather than *P. gingivalis*[24]. The microflora of acute periodontal diseases was reported to be dominated by *T. denticola* with clinical presentation of

acute necrotic periodontal diseases along with orange complex organisms (*F.nucleatum and P. intermedia*)[24].

Introducing a culture-independent 16S rRNA sequencing opened a new horizon in search of the pathogens and revealed many more species that were not usually detected in the cultures[25]. Roberts et al. (2015) emphasized the survival of the fitted phenotypes in the 'oral microbial community' setting. A combination of newer technologies in exploring the various locations of oral cavities and the shifting of paradigm in understanding the pathophysiology of periodontal disease was able to open a new era of "Polymicrobial symbiosis and dysbiosis."[26,27,28] Hajishengallis and Lamont (2012) reported that the pathophysiology of periodontal diseases is an example of polymicrobial synergy and dysbiosis. This concept starts with the healthy periodontium associated with commensal microbiomes. "Keystone pathogen"[27](see below) then invades this biofilm changing its equilibrium negatively towards pathogenicity. This transition is regulated by the host's inflammatory process indicating a mutual interaction between bacterial microbiome and host factors[28].

2.3 **Colonization of subgingival sites**

As described by Listgarten et al. (1978)[29], there may be a pattern in building the biofilm from the early colonizers to the late arrival of the key pathogens. Regardless, periodontal bacteria adhere to epithelial cells in the gingival crevice and to other bacteria, which are already present to build a community (subgingival plaque). Adhesins are often associated with fimbriae on the bacterial surfaces. For those bacteria outside of the gingival sulcus will either proliferate or translocate into the sulcular areas for better survival. Some species, such as spirochetes, can use

their ability to be motile via axial filaments that can reach the sulcular areas and deeper sites by chemotactic attraction. [30] These are anaerobic microorganisms and cannot grow or even die in the presence of oxygen. These anaerobic microorganisms can be further divided into obligate anaerobes, facultative anaerobes and aerotolerant organisms. Strict anaerobes cannot survive in the presence of oxygen level of 0.5% whereas, moderate anaerobes can survive at the level of 2-8% by producing superoxide dismutase to protect them from oxygen.[31] Facultative anaerobes can survive or grow with presence or absence of oxygen. However, there is an aerotolerant microorganisms that can survive only but cannot grow in the presence of oxygen.[32] These anaerobic microorganisms are fastidious, it is hard to culture and isolate them or even difficult to recover them from the infected sites. Infections from these anaerobes can be serious and the common bacteria involved in the anerobic infections are gram-negative rods such as *Porphyromonas, Fusobacterium, Prevotella* and *Bacteroids*.[33]

P. gingivalis and *A. actinomycetemcomitans* are well-known for their ability for infiltrating into gingival epithelial cells[34,35]. The intra-cellular areas protect these from the immune surveillance and cause the cells to release cytokines. Even though the exact mechanism of penetration and translocation processes from the epithelium to gingival connective tissues is unclear[36], utilization of fimbriae onto ß-1 integrin receptors and various surface proteins by *P. gingivalis* and *T. forsythia* were elucidated[37]. Invasion leads to inter-cellular pathway that may involve various mechanisms to spread within the tissue such as membraneous projection, endocytic recycling pathway, which are more advantageous as those translocation are not detected by immune-surveillance[37]. Along with *P. gingivalis, T. denticola* invade into the epithelial layers and pass through to the connective tissue areas causing layers of tissue damages by strong

proteases. Those proteases include Dentilisin targeting the junctional proteins such as E-cadherin and occluding in the junctional epithelium[7]. Dahlen et al. (2019) described the invasion process as innocent where not immunological process as long as there is a balance between commensalization and colonization[38]. In inter-cellular pathway, the initial stage of inflammation weakens the tight junctions between junctional epithelial cells which may be opportunistic for motile pathogens to penetrate into. In intra-cellular pathway, there were evidence showing multiple species were residing in the cells such as fimbriated *P. gingivalis, T. forsythia, P. intermedia* and *C. rectus*[38]. Invasion may also occur unintentionally during dental procedures inducing traumatic ulceration in gingival connective tissue or by release of variety of proteinases produced by the pathogens creating a pathological ulceration that can be a great source of invasion.

2.3 Keystone pathogen hypothesis

Koch's postulates defining the criteria for a single pathogenic bacterium causing disease do not apply well in periodontology. Defining when periodontal pockets are in active stage of breakdown is difficult if not impossible. In addition, the presence of periodontal pathogens in the pockets as such does not prove that they are causative factors of periodontitis. Therefore, Socransky (1977) came to his own postulation in the context of the periodontics to make it more suitable for understanding the role of periodontal pathogens in periodontal diseases[39]. He stated that a periodontopathogen must be associated with the diseased periodontal sites (Association). Once eliminated, the state of disease must improve to the state of heath (Elimination). In addition, the pathogen must be able to elicit immune reaction (Host Response), cause similar disease in animal experimentation and contain virulence factors. As Socransky published the paper on 'the complex theory' in 1998, the paradigm shift was initiated from the non-specific plaque hypothesis

to the 'specific plaque hypothesis.'[6] The pathogens were classified by the level of association of periodontitis with different phenotypical variations such as onset and progression. The early colonizers of bacteria such as cocci and rods were labeled as 'green' and 'orange associated complexes. They usually adhere to the pellicles to form an initial foundation for the biofilm complex that it can build upon. The orange-associated complex is not same as the orange complexes in the sense that the orange complex is considered to be 'bridging' species between the early colonizers and the red complexes with higher pathogenic capacity. The red complexes are the late addition to the biofilm that communicate with the existing colonizers. Bartold et al.(2020) reported that these red complexes are not an additional arrival but rather these are part of the commensal flora.[40] It is just the environment of the periodontal pockets that flourishes these bacteria and provides adequate nutrients and metabolic requirements that will satisfy the emergence of red-complex bacteria. Therefore, one of the Socransky postulations (1977), the 'elimination' is always being challenged. There is no periodontal treatment that can completely eliminate the periodontal pathogens although their number are significantly reduced. Hajishengallis et al. (20120 claimed certain bacteria, "Keystone pathogens ", particularly *P. gingivalis* of the red-complex can drive the community of biofilm towards dysbiosis[41]. *P.gingivalis* has been known to be dependent on commensals[42]. However, even at low numbers (<0.01% of the total microbiota), *P. gingivalis* was able to transform the community to pathogenic state.[41] It means even a keystone pathogen needs not only an adequate environment but also the commensals proving their symbiosis relationship.

2.4 Dysbiosis

Dysbiosis is a term that explains the shift in periodontal biofilm that leads to increased pathogenicity. The authors defined dysbiosis as "a state of imbalance in the relative abundance or

influence of species within a microbial community associated with inflammatory disease" [28]. In dysbiosis, there is uncontrolled battle between the biofilm for a survival and expansion and the host response to contain or eliminate it. The level of communication among the various microbiota with the whole oral biofilm is increased and therefore the pathogenicity of the biofilm will enhance resulting in a more pro-inflammatory response from the host including hyper-response of epithelial and immune cells to the bacterial infiltration with increased secretions of cytokines[28]. Upon the hyper-release of cytokines, *P. gingivalis* can trigger 'chemokine paralysis' as a protective mechanism for the survival of the community. Enzymes from periodontal pathogens such as *P. gingivalis* (gingipain), *T. forsythia* (karilysin) and *P. intermedia* (InpA) can manipulate and over-ride the host immune response with 'subversion of complement'. Complement system has multiple functions in the immune system. It forms the membrane attack complex (MAC: C5b-C9), opsonization (C3b) and creating powerful chemotaxis by producing C3a and C5a. The complement system will not work efficiently with these microorganisms[28]. Also, the enzymes from the periodontal pathogens such as gingipains can also manipulate the neutrophils and break the TLR-2 pathway to cease the inflammation process of neutrophils. In this process, *P. gingivalis* does not only protects itself but also others in the community.

The concept of 'Polymicrobial synergy and dysbiosis' by Hajishengallis et al.(2012) is not an entirely new concept, but incorporates significantly more host tissue response in development of pathogenic biofilm[27]. For example, it is the local environment facilitates the red complex bacteria to establish themselves as keystone pathogens through production of gingipain, fimbriae, and other virulence factors. Also, the production of lipopolysaccharide is influenced by host temperature and the level of hemin. This evidence provides the fundamental understanding of these red complex bacteria transforming into the pathogenic keystone bacteria, not because they would

like to cause disease but rather to follow their physiological condition to survive and expand. These community members including 'accessory pathogens' would have their own requirement for survival and expansion among their such as heterotypic community adhesion requirement, physiological compatibility (at least not inhibitory to each other) and ability to coordinate the defense against the host immunological attack to preserve their community[28].

2.5 Peri-implantitis: definition of diagnosis

2.5.1 Peri-Implant Health

Histologic characteristics of healthy peri-implant mucosa are significantly different from those around teeth. The portion of peri-implant mucosa facing the oral cavity is covered by keratinized epithelium and the opposite side (implant side) has two parts, 1. barrier epithelium (equivalent to the junctional epithelium of the gingiva) and sulcular epithelium, 2. Connective tissue in direct contact with the implants/abutments. [43] In the connective tissue lateral to the barrier/sulcular epithelium, vascular anastomosis-like collection enriched the area away from the adhesion zone. Moon et al. (1999) reported in their dog study that the adhesion zone has two layers: inner (40 μm) and outer (160 μm).[44] A large number of fibroblasts exist in the 'inner zone' in intimate contact with implant surfaces and the 'outer layer' is mainly comprised of collagen fibers. The authors also reported that compared to the tissue composition around teeth, there are more collagen with fewer fibroblasts in the peri-implant tissues.[44] Those collagens were also characterized by their orientation being parallel to the implant surfaces.

Berglundh et al. (1991) examined the oral mucosa formation around the implants reported peri-implant mucosa is consisted of two layers: keratinized oral epithelium (external side) and thin

barrier epitheium (internal side).[45] This thin barrier epitheium (peri-implant junctional epithelium) terminated 2 mm from the most coronal part of the marginal gingiva and 1.0-1.5 mm away from the crestal bone. Abrahamsson et al. (1996) also confirmed similar findings in comparative study in the dog. In histological observations, authors reported that a junctional epithelium ranged about 1.6 – 2.3 mm and the distance between the apical area of junctional epithelium and the crestal bone was about 1 mm. The authors reported that those tissue between the junctional epithelium and the crestal bone was more like a scar (i.e. dense collagen fiber with few cells and vascularity).[46]

Peri-implant health is defined as "absence of clinical signs of inflammation (erythema, and edema)" accompanied by no tissue response (i.e. bleeding and/or suppuration) upon probing[47]. These requirements aligned with ones with periodontics literature.[48] However, one must take consideration understanding the nature of soft tissue around the implants (see above). Due to its inherent weakness around the implants, probing through soft tissue seal at the implants neck area may induce bleeding due to mechanical trauma mimicking biofilm-induced inflammation[49]. Periodontal probing around the implants may be more sensitive to force in comparison to that around the teeth[50]. In general, probing around the implants can be challenging as the abrupt change of contour from the implant fixtures to supporting crowns resulting in aberrant angulations of probe. Even though it may be debatable, the probing depth around the implants should be ≤ 5 mm.[43]

Radiographic analysis is another critical element to define the health status of peri-implant tissues. Ideally, there must be a reference to compare to witness an active and progressive bone loss around implant fixtures. In this context, initial bone remodeling must be considered as a normal physiological change instead of pathological changes. Therefore, bone remodeling is acceptable as long as it is within 2 mm.[51,52] Clinical evaluation such as the absence of biofilm, no

bleeding on probing along with no progressive bone loss after initial loading with not more than 2 mm are considered to define the peri-implant health.

2.5.2 Peri-Implant Diseases

2.5.2.1 Peri-Implant Mucositis

Clinical signs of inflammation, including red, swelling and soft tissue consistency around the implants without bone loss are a common feature of case definitions of peri-implant mucositis.[47] The bleeding pattern when probed must be more than a single dot-like pattern and suppuration can be one of the clinical signs. Dot-like bleeding pattern can be evident in traumatic probing around the implants due to abrupt changes of emergence profile of crowns. Therefore, other visual examinations must be consistent with bleeding on probing as a confirmatory procedure instead of "Rule-in" or "Rule-out" of diagnostic procedures. In the absolute absence of other signs of inflammation around the soft tissue, then the signs of bleeding on probing must be interpreted with a critical mind to rule out a traumatic probing incidence. As the progressive increase of pocket depths can be another feature of the peri-implant mucositis, the baseline probing depths are important to establish this diagnosis. Since progressive bone loss is not a characteristic of peri-implant mucositis, it is important to record the marginal bone changes from the baseline after loading. Bone remodeling during the first year after loading should not be considered as an element of peri-implant mucositis. The accepted value for initial bone changes should not exceed more than 2.0 mm.[47]

Histologically, the biopsy study showed that inflammatory cell infiltrate showed the dominance of B-cells as well as PMNs around the peri-implant mucositis areas.[53] Karatas et al. (2020) investigated hypoxia-related tissue changes and compared them in control (healthy),

periodontitis, peri-implant mucositis and peri-implantitis.[54] The target markers were ‘fibroblast’, ‘inflammatory cells’, ‘hypoxia-inducible factor (HIF-1a)’, ‘prolyl hydroxylase’, ‘matrix metalloproteinase (MMP)-8’, ‘tissue inhibitor of MMPs (TIMP)-1’, ‘cyclooxygenase(COX)-2’ and ‘inducible nitric oxide synthase levels’. Biopsies from 15 patients of each group had been examined and peri-implant mucositis had higher fibroblasts and lower inflammatory cells compared to periodontitis and peri-implantitis. HIF-1a, COX-2 and iNOS had significantly lower values in peri-implant mucositis than ones in periodontitis and peri-implantitis. The authors concluded peri-implant mucositis had disease status between the healthy control group and periodontitis/peri-implantitis groups. [54]

2.5.2.2 Peri-Implantitis

Renvert et al. (2018) defined the peri-implantitis and reported that most frequent clinical signs to define peri-implantitis were ‘bleeding on probing’ and ‘bone loss’.[47] The key element is those two signs must accompany together after initial healing and function. In case of lacking baseline, then implant shoulder level was the reference point to measure the amount of bone loss. However, it is true that different cut-off values for bone loss were commonly seen therefore, the prevalence of peri-implantitis may vary naturally. One of the challenges the dental community has faced is the lack of standardization of diagnostic factors and values for a clear case definition in achieving a universal and unanimous diagnosis. In the systematic study and meta-analysis, Ramanauskaite et al.(2016) reported that there is no uniform definition that can diagnose a case in a universal language.[55] Accepting the variations of different clinical criteria, the consensus characterized the peri-implantitis as a progression from its predecessor; peri-implant mucositis with clinical signs of inflammation ‘AND’ progressive bone loss. [56]

The bone loss around the implants has been in debate to explain the phenomenon as a physiological realm or a pathological expression. Multiple studies supported that bone loss less than 2.0 mm is to be considered as normal accounting for a physiological remodeling around foreign body. [52,57,58] Not only is the amount a variable, but also the rate/timing of bone loss is also another variable. Fransson et al. (2005) concluded that bone loss around the implants is not a linear progression.[59] Therefore, it is critical to monitor the bone loss or changes to identify if it is a true peri-implantitis case. Other clinical signs and symptoms must accompany by observed patterns of bone loss to recognize the peri-implantitis. It is reported that there are no models or flow-chart to predict the progression of the disease. [47] Hence, the knowledge on periodontitis is applied to peri-implantitis since periodontitis is known to have a feature of 'slow and burst' rate of disease progression. [60] Socransky et al. (1984) also reported that individual sites of periodontitis inpatients had a different rate of breakdown in a longitudinal study. Some individual sites had a faster breakdown than others. The authors concluded that the periodontitis progresses as "recurrent episodes" with a period of rapid breakdown followed by periods of extended quiescent period. However, the authors added that those burst of destruction can occur with high frequency during some specific times of one's life. [61]

Marginal bone loss in peri-implant tissues is usually detected by radiographic methods. However, these radiographic analyses can mislead a clinician as those are not standardized and the degree of variations is extremely high due to multiple factors such as equipment, operators, patients, and locations of implants. Therefore, it is important to understand those variations in literature and must consider the standard deviation in measuring the amount of bone loss. Sanz et al. (2012) suggested, in general, any bone loss beyond the measurement error (less than or equal to 2 times of standard deviation) or 2 mm of marginal bone loss to be considered as an indication

of peri-implantitis cases.[58] In general, baseline radiographs are therefore very important for comparison to obtain the evidence of progressive bone loss. However, it is not uncommon to accept patients with implants who cannot provide the baseline radiographs. Renvert et al. (2017; World workshop by American Academy of Periodontology) provided a case definition for day-to-day clinical practice for the cases lacking the baseline radiographs. The authors suggested that suspected bone loss of three (3) or more mm and/or probing depth of 6 mm or more with bleeding represents peri-implantitis. [47] It is suggested to make annual assessment for marginal bone loss to calculate the yearly rate of bone loss.

Froum et al. (2012) proposed classification of peri-implantitis based on the percentage of bone loss and pocket depths into three categories: 'early', 'moderate' and 'advanced'.[62] All categories have to express 'bleeding and/or suppuration on probing'. Early peri-implantitis has pocket depths $\geq$ 4mm and bone loss $<$ 25% of the implant length. Moderate peri-implantitis has pocket depths $\geq$ 6mm and bone loss ranges between 25% to 50% of the implant length. Advanced peri-implantitis means pocket depths $\geq$ 8mm with the bone loss $>$ 50% of implant length. [62]

Schwarz et al. (2007) described different configurations of bone defects in 'ligature-induced peri-implantitis' in dogs. Schwarz and coworkers categorized them in Class I and Class II. Class I is intrabony defect with a dehiscence, partial to complete cratering or combination of two resulting in subclassifications from Class Ia to Ie for various intrabony defect configuration.[63] Class II is a horizontal bone loss around the fixture without intrabony and vertical components in bone loss. Most common defect was Ie with more than 50% of human subjects and 80% in dog samples.

Further to this, Zhang et al. (2014) reported by examining 83 human subjects who received implants for over-dentures.[64] The authors categorized the bone defects into 5 different types; Type

1-5 (type 1: saucer-shaped, type 2: wedge-shaped, type 3: flat or no vertical component, type 4: undercut and type 5: slit-like). The authors reported type 2 (wedge-shaped) and type 3 (flat type) were the most frequent bone defects. However, awareness of different types of bone defects would not dictate different types of disease management or predict the success of peri-implantitis treatment.[65]

Many studies reported that perio-implantitis lesions showed more prominent presence of neutrophil and other granulocytes reflecting the signs of more acute inflammation.[66 67] It, clinically, also reflects that by showing more rapid progression than the periodontal diseases. Furthermore, in the most apical part of the lesions, there is no pocket epithelium, separating biofilm from the inflammatory infiltrate (i.e. bacteria are outside of the body in periodontitis while they are inside of the body in per-implantitis inflammatory lesions). Therefore, peri-implantitis inflammatory lesions extend to bone which is not the case in periodontitis.

2.6 Microbiology of peri-implantitis

There are consistent findings in the literature that the peri-implantitis lesions harbor various but somewhat usual pathogens of periodontitis[50]. It makes sense because the implants are being placed in the oral cavity and within a short period of time of 1-2 weeks, the microbiome starts initiating its formation of the biofilm in the sulcus of the implant and reaches its stability within 3 months[68]. Mombelli et al. (1987) reported that the pathogen profiles found in peri-implantitis were similar to those found in periodontitis and there was a transition from a Gram-positive facultative species to a Gram-negative anaerobic species within biofilm[69].

Microbiomes in biofilm are resistant and protected from the external stimuli or attack by having a protective and nutritional layer of the extraceullar polymeric substances.[70 71] Also, Microorganisms in the biofilm can have symbiotic and synergistic relationship or competitive

relationship. In oral cavity, adhesion of bacteria to the tooth surface or implant surfaces is aided by acquired pellicile of surface proteins and abundant polysaccharides on the various surfaces with van der Waals forces, covalent bond and ionic bonds.[72] The processes of adhesion on teeth and implants are similar to each other. However, there may be difference in the surface characteristics and properties that may result in the different affinity to the surface among microorganisms.[73 74] Transformation of the nature of biofilm from health to pathogenic one reflects the microbiomic transition from the commensal microorganisms to the ones with keystone pathogens and other associated microorganisms.[75 41]

Healthy teeth and implants harbor similar microflora of gram-positive, aerobic cocci and short non-motile rods.[76] The early colonizers in either tooth or implants similar and those include *Actinomyces* sp. and *Streptococcus* sp. such as *Streptococcus oralis*, *Streptococcus infantis* and *Streptococcus sanguinis*. [68] Especially, healthy implants sites showed the presence of *Streptococci, Veillonella, Rothia* and *Haemophillus* species and *Actinomyces* species.[68] Healthy sites can harbor pathogens knows as keystone pathogens, *P. gingivalis*. However, the level of presence is low and if so, there are no signs of inflammation at the clinical level.[8 42]

The nature of periodontal and peri-implant diseases can not be elucidated without involvement of gram-negative and facultative or obligate anaerobi bacteria and therefore these are infections diseases.[77] Red complex (*P. gingivalis, T. forsythia, T. denticola*), *A. actinomycetemcomits*, and orange complex (*F. nucleatum, P. intermedia, P. negrescnes and P. micros*) bacteria are considred to be pathogens in development of periodontitis and peri-implantitis.[5 50 77] Among these pathogens, *P. gingivalis* is labelled as a keystone pathogen that shifts the nature of biofilm from non-pathogenic to pathogenic including the contribution of the commensal microorganisms,[28]

Compared to teeth, microflora around the implants are considered to be more heterogenous and anaerobic than healthy implant sites.[78,79] A majority of studies support that peri-implantitis has higher prevalence of red-, orange-complex and enteric rods [78,80] Additionally, many studies show the some degree of heterogenous types of microbiomes in peri-implantitis including *Filifactor alocis, Treponema maltophilu, Freitibacterium fastodisu, Parviromonas micras*, *Campylobacter* species, *F. nucleatum* and *A. actinomycetemcomitans*. [81,82,83] Even though *Streptococcus* sp. were considered to be the early colonizer and associated with healthy sites, levels of *Staphylococcus aureus* and *S. epidermis* were increased in peri-implant disease.[79] In-vitro study of roles of *Staphylococcus* in peri-implantitis development found out there were competitive relationship between *S. aureus* and *S. epidermis.*[84] The authors assessed the submucosal biofilm model in vitro on titanium and found out *S. aureus* outgrew *S. epidermis* when they were added together. All other original species such as *P. gingivalis, T. forsythia, T. denticola, A. oris, F. nucleatum, P. intermedia, C. rectus, S. oralis, Veilonella dispar* and *Streptococcus anginosus* remained at the same level.[84] Renvert et al. (2014) stated that some studies identified *S. aureus, Eurobacteriae, Candida albicans* and *Pseudomonas aeruginosa* in peri-implantitis biofilms that are not usual species in biofilm around teeth.[85]

In comparison study by Shibli et al. (2008), the composition of supragingival and subgingival plaque biofilm on the diseased implants showed significantly higher proportion of red complex organisms in the peri-implantitis group in both supra- and subgingival areas. The authors reported more prevalent red complex organisms compared to the healthy implants[86].

Kroger et al. (2018) reported the peri-implantitis microbiota in-network analysis[87]. Having different severity levels, the authors showed the relationship between the clinical measurements to the degree of dysbiosis. The authors reported that the outcomes were similar to the conventional

findings that the peri-implantitis lesions were harbored by Gram-negative and anaerobic taxa. The authors reported the two species representing the health around the implants were *Streptococcus* and *Veillonella* and the diseased site harbored anaerobic (mostly gram-negative) bacteria.[87]

The microflora of peri-implants sites is difficult to analyse due to high levels of variations among individuals and culturing/identification of bacteria. There is significant level of heterogeneity in the literature, but general consensus shows red/oranges complex with *S. aureus* are dominant species. Similar to periodontal diseases, the primary etiology is biofilm and its byproducts in development of peri-implantitis. Therefore, it is important to understand the microflora and its nature around the peri-implant sites.

2.7 Prevalence of peri-implantal diseases on implants and subjects

In inquiry of the prevalence of peri-implant diseases, it is commonly accepted that there is lack of a clear definition of peri-implants diseases that may result in inaccurate and inconsistent measurements of prevalence.[76, 88, 89, 90 ,91] First definition of peri-implant disease was introduced by Albrektsson and Isidor in consensus report of the 'Proceedings of the First European Workshop on Periodontology' in London, 1994.[92] The authors stated that the peri-implant disease is "a collective term for inflammatory processes in the tissues surrounding an implant". However, those definitions became too broad to be useful in the literature, resulting in a high level of heterogeneity of definitions.

Derks et al. (2015) reported the systematic review and meta-analysis on the prevalence of peri-implant mucositis and peri-implantitis in 2015. [88] The authors reported the most significant issue in selecting articles was a lack of standardizations in designing studies and reporting the results. The authors reported prevalence was in a range of 19-65% for peri-implant mucositis and

1-47% for peri-implantitis. This is most unbiased analysis by selecting samples in most random ways in current authors' view.

Zitzmann et al. (2008) reported the prevalence of peri-implant diseases reviewing cross-sectional and longitudinal studies with a minimum of 50 subjects with more than 5 years of follow-up.[93] Prevalence of Peri-implant mucositis was at 80% of the subject and 50% of the implants while peri-implantitis was 28-56% and 12-43% of in the subject level and implant level, respectively. The authors advocated having two separate analyses at the patient level and the implants level with extent of disease for each subject. The authors reported that there were only a few studies had provided the prevalence of peri-implant diseases. More cross-sectoinal studies with larger sample sizes (i.e. 100-500 patients with implants therapy) in private/public dental clinic rather than university clinics were recommended.

Lee et al. (2017) reported in his systematic review that there was a high level of heterogeneity in diagnostic criteria within literature.[89] The authors included studies with a minimum of three years of follow-up and a minimum of thirty implants or patients. Mean peri-implant mucositis was 29.48% and 46.83 at implant-level and patient-level, respectively, and the mean peri-implantitis was 9.25% and 19.83% in implant-level and patient-level, respectively. The interesting finding in this report was that the included studies had a very high degree of variation in determining the marginal bone loss.

Establishing a baseline of the diseases is a pre-requisite to identify, understand and plan for the treatment/maintenance. However, the high level of heterogeneity in the literature makes it more difficult to define a diagnosis and, therefore prognosis. Naturally, creating a prevention protocol can be very challenging as there are no set guidelines of defining the diseases yet, to measure the effectiveness of therapies in various clinical settings.

2.8 **Pathogenesis of peri-implantitis**

Peri-implantitis is an inflammatory process that influences on the health of soft and hard tissues around the implant fixture and supra-structures of the fixture. [93] Peri-implantitis is similar to periodontitis in the sense that its scope of diseased areas includes both soft and hard tissues at the site of interest. The exposed surfaces will naturally harbour the biofilm by infiltration and attachment of glycoproteins producing pellicle layers to be able to initiate the biofilm formation. This process is somewhat similar to that of periodontitis by having gram-negative anaerobes. [65] However, Leonhardt et al.(1999) reported that even though putative periodontal pathogens such as *P. gingivalis, P. intermedia, P. nigrescens* and A. *actinomycetemcomitans* were found in 60% of the cases, there were some uncommon periodontal pathogens present in the peri-implantitis sites such as *Staphylococcus spp*., enteric and *Candida spp*. in 55% of the peri-implantitis sites[94]

Vasculatures within the soft tissue (gingiva and/or mucosa of the barrier epithelium and connective tissues) at the interface of biofilm dilate and become more permeable for infiltration of PMNs (Polymorphonuclear neutrophils), especially neutrophils. One of the major components of the extracellular matrix in the tissues is collagen keeping the healthy and optimal integrity of tissue structure around the implants. However, as the diseases progress, the density of collagen decreases by bacteria and host immune response. Production of collagenase is one of the immunological responses of the human body as well as methods of destruction driven by bacteria. Once cytokines are released at sites, then fibroblasts and osteoblasts release collagenase and cause the neighboring tissues inadvertently. The loss of collagen in the underlying connective tissue below barrier epithelium is a typical sign of the presence of inflammation and loss of collagen opens the space for infiltration of lymphocytes. Especially, T- and B-cells migrate into the peri-implant mucosa.

Lindhe et al. (1992) reported in the comparative experimental animal study with beagle dogs that one common feature of periodontitis and peri-implantitis was the loss of connective tissues.[95] However, the difference was the severity of connective tissue damage was more pronounced in the peri-implantitis compared to periodontitis. The authors also added that compared to periodontitis sites, there was significantly higher number of neutrophils and osteoclasts in peri-implantitis sites. The inflammatory process eventually involves osteoclastic activity on the alveolar bone around the implants resulting in marginal bone loss. Interleukin-1, Interleukin-6, Tumor Necrosis Factor-Alpha and Prostaglandin E_2 mediate the bone loss stimulating osteoclasts. Intense infiltrates of inflammation migrate into the sites producing more cytokines for marked inflammatory responses. Fibroblasts also upregulate the production of pro-inflammatory mediators and MMPs in peri-implantitis disease progression. [96] More specifically, the levels of MMP-7 and MMP-8 were more elevated in gingival crevicular fluids in peri-implantitis and the investigators reported those MMPs were the main cause for tissue destruction.[97] Compared to pro-inflammatory mediators such as Interleukin-8, anti-inflammatory mediators such as Interluekin-10 were reported to be low in peri-implantitis lesions.[98]

There are still many attempts to find specific genotypical or systemic immunological markers for an indication of peri-implantitis and its severity without success.[99] The current research is focused on analyzing peri-implant crevicular fluid (PISF), which is similar to gingival crevicular fluid (GCF) to natural teeth.[100] Receptor activator of NF-kappa b ligand (RANKL)-osteoprotegerin (OPG) system may be an indicator of peri-implantitis disease and severity as OPG plays a decoy receptor for RANKL to inhibit RANK-RANKL binding.[101] Rakic et al. (2014) reported useful biomarkers for detecting peri-implantitis in the context of pathogenesis of peri-implant diseases. The authors reported the prognostic roles of RANK, RANKL and OPG and their

concentrations reflect the osteoclastic activity around the implants and significant higher concentration of RANKL in peri-implantitis sites.[102] Costa et al. (2018) reported the importance of osteoclastogenesis and investigated the level of RANK, RANKL, OPG and RANKL/OPG ratio. The authors reported high levels of RANKL and RANKL/OPG ratio in mucosal biopsy and its correlation to peri-implant mucosal inflammation.[103]

Histopathologically, Berglundh et al. (2011) reported that periodontitis and peri-implantitis might have some differences.[66] First of all, the authors pointed out there are only a few studies that had involved human biopsy in histological studies. Among the human biopsy studies, Berglundh et al. (2005) reported that B cells and plasma cells were the dominant cell types in chronic and aggressive periodontitis with less than 5% of T-cytotoxic cells, neutrophils and macrophages.[104] More intense expression of inflammatory cell infiltrate was evident in peri-implantitis in the human biopsy studies. Inflammatory cell infiltrates contained B-lymphocytes more dominant over T-cells. It also reported a high number of elastase-positive cells close to the central and towards the pocket epithelium. Commonly, a more apical location of pocket epithelium was found, and almost the entire inflammatory cell infiltrate occupied the connective tissue zone in the peri-implantitis lesions. In both cases, the dominant cell types were plasma cells and lymphocytes. However, neutrophils and macrophages were more abundant in peri-implantitis lesions than periodontitis lesions. Location of these neutrophils and macrophages were found in pocket epithelium zone in periodontitis but, in peri-implantitis lesions, those PMNs were also found in peri-vascular areas. There is no pocket epithelium at the apical areas of inflammation being more vulnerable due to direct contact with those pathogens. The observation on ligature-induced diseases in the experimental studies showed that the reversal process of diseases was more prominent in periodontitis. Periodontitis lesions did show ‘self-limiting’ capacity to limit the

disease progression having a small zone of non-infiltrated connective tissue zone keeping the inflammatory cell infiltrates away from the bone. In peri-implantitis tissues, there was no connective tissue to prevent inflammatory cell infiltrate from extending towards the bone. Zitzmann et al. (2004) reported "spontaneous progression of experimental peri-implantitis" where once 40% of bone support disappeared, the majority of implants still progressed further even after ligatures were removed.[105]

Presence of keratinized tissue and traumatic occlusion were also reported to differentiate the histological variation between two diseases by showing more pronounced destruction around peri-implantitis lesions. [106] In the histological evaluation of gingival and peri-implant tissue samples, the level of fibroblasts, inflammatory cells (neutrophils, lymphocytes, eosinophils and macrophages) and other factors (HIF-1a, PH, MMP-8, TIMP-1, COX-2 and iNOS) via immunohistochemistry were lowest in healthy control group. These parameters were higher in peri-implantitis compared to peri-implant mucositis.[54] The authors reported that peri-implantitis and periodontitis had similar cellular profiles and counts. The healthy group had the lowest inflammatory cell density, whereas periodontitis and peri-implantitis had similar levels to each other, being higher than the healthy and peri-mucositis groups. HIF-1a showed the highest level in periodontitis and peri-implantitis. The authors concluded that within the limitation of their parameters of markers, periodontitis and peri-implantitis shared the similarities in cellular components in the connective tissues.

2.9 **Risk factors and indicators in peri-implantitis**

Risk factor is an epidemiological term to define the correlation, not necessarily causation of the disease or infection. A risk factor is a variable associated with increased risk or infection. Risk determinant is another term that is often used as a type of risk factor. It includes factors that

cannot be changed or modified such as sex, age, or race. Risk indicators are probable risk factors that have been identified in cross-sectionsal or correlational studies but not confirmed through longitudinal studies. To define them in the field of periodontology, the consensus report in 1996 in the Annals of Periodontology defined the risk factors and risk indicators as "an environmental, behavioral, or biological factor that, if present, directly increases the probability of a disease (or adverse event) occurring and, if absent or removed, reduces that probability.[107] Risk factors are part of the causal chain or expose the host to the causal chain" and "probable risk factor that has not been confirmed by carefully conducted longitudinal studies", respectively[107].

Heitz-Mayfield et al. (2008) reviewed those potential risk factors to provide a guideline for clinical practices[91]. Due to the nature of the field, the majority of these studies were cross-sectional studies utilizaing various diagnostic tools and methods such as peri-implant crevicular fluids with saliva analyses, radiographic analyses, implant mobility and suppurations to identify the presence of the diseases. The main tools to provide evidence for the disease presence were the increasing probing depth (0.25N), the presence of BOP and suppuration along with radiographic analyses. The authors reported that the risk indicators with strong evidence included poor oral hygiene, history of periodontitis and cigarette smoking.

Gurgel et al. (2017) reported high gingival index, medications and multiple implants were the associate factors in the cross-sectional study[108]. In this study, the authors claimed that the peri-implant diseases were characterized as asymptomatic and usually incidentally found during the recall examinations. Peri-implant mucositis was defined as bleeding on probing with gingival bleeding and/or suppuration. Peri-implantitis was defined as the PD is 5 mm or more with tissue response with bleeding on probing and/or suppuration with apparent bone loss.

Marrone et al. (2013) reported in the retrospective study, the risk factors of peri-implantitis in the Belgian populations.[109] Unlike the risk factors of periodontitis, the authors actually could not find smoking as one of the risk factors, but that was likely due to the significantly lower number of smokers in the subject pool. The patient-related factors included total edentulism, hepatitis, active periodontitis, age of 65 and up, generalized plaque accumulation (more than 30%), diabetes, history of periodontitis, smoking, and lack of dental visits. The risk factors related to the implants were 'bone-level vs. tissue-level, 'rough surfaces,' '10 and more years of function' and 'over-denture type of prosthesis.' The authors elucidated these risk factors due to ageing process associated with impaired-vision, diminished dexterity in maintenance and lack of motivation in plaque control. Also, the patient ageing process inevitably associate with more occurrence of illness, wound healing capacity and systemic conditions such as hepatitis and diabetes. In regard to the implants-related risk factors, the presence of microgap at the subgingival level for bone-level implants and rough surface create more challenge in achieving optimal plaque control.

Even though the numerous studies supported the prevalence of these diseases with significant figures yet, the risk factors of the peri-implant disease have not been truly identified due to its characteristics of the disease. More prospective longitudinal studies in regard to the risk factors will shine the lights in finding risk factor of peri-implant diseses as the history of periodontitis, poor oral hygiene and smokings were risk indicators with strong evidence[91].

Chapter 3: Aims and hypotheses

As dental implants are being a part of routine dental care, the prevalence of peri-implantitis is increasing. There are numerous studies to explore the microbiome on the surface of dental implants. However, to this date, there have been no published studies in analysing and visualizing the morphotypes of those microorganisms on the clinically failed and removed implants due to peri-implantitis.

The aim of this study

To directly visualize and quantify bacterial morphotypes on the surface of biofilm of failed implants removed due to peri-implantitis.

Hypothesis

There will be transition from coccoid- and rod-dominant morphotypes to more filaments and spirochetes/spirilles with advancement of bone loss on the failed implants.

Chapter 4: Materials and Methods

4.1 Sample collection and processing

4.1.1 Sample Collection

The specimens were collected at the Graduate Periodontics Clinic in the University of British Columbia, Vancouver, Canada, during routine patient care (University of British Columbia ethics protocol #H15-01881). Implants removed due to peri-implantitis were placed in physiological saline solution (0.9% NaCl) and immediately transported to the laboratory.

4.1.2 Sample Fixation

The biofilms on failed dental implants were fixed with 2.5% glutaldehyde in 0.1M PIPES buffer (pH 7.4) for 30 minutes at room temperature. After fixation, the specimens were rinsed with 0.1M PIPES buffer solution three times to remove any unreacted glutaraldehyde. The specimens were then transported to the UBC Centre for High-Throughput Phenogenomics for processing and imaging.

4.1.3 Critical point drying, mounting and coating for scanning electron microscopy

Samples were dehydrated in a successive increasing concentration of ethanol (EtOH; Electron Microscopy Sciences, Hatfield, PA) for 5 minutes each at 50%, 60%, 70%, 80%, 90% and 3 times 5 minutes at 100%. After dehydration, the samples were transferred to ‘Tousimis Samdri-795’ critical point dryer (Tousimis Research Corporation, Rockville, Maryland, U.S.A).

Once fixed, dehydrated, and dried, the samples were ready for the mounting process. Two samples were mouted with glue and the remaining twelve (12) samples were secured by mini screws allowing two sides to be analyzed by scanning electron microscopy. Samples were then coated with 8 nm of iridium using the Leica EM MED020 Coating System (Leica Microsystems, Wetzlar, Germany).

4.2 Scanning Electron Microscopy

Each sample was examined using the scanning electron microscope (Helios NanoLab 650 Focused Ion Beam SEM, Oregon, USA). The implant samples were viewed under various magnifications from 65x to 50,000x. The main images of samples were taken at the magnification of 5000x for a sole purpose of morphotype quantification. Any special characteristics in the biofilm were imaged at the higher magnification of 10,000x or 50,000x.

Additoinally, random areas of biofilm were chosen to obtain cross-sectional views of biofilm. In a scanning electron microscopic analysis, electrons that are released to create the secondary electron have low-mass eletron to obtaine the surface images with high quality. Helios NanoLab 650 has 'Focused Ion Beam (FIB)' function where, instead of electrons, a beam of ions is used. The focused ion beam can modify the surface with controlled energy to be able to create a cross-sectonal view of the sample. Controlling a level of energy and intensity of beam result in the different depth of cross section with high precision in nanometer level.[110] The images were taken from the mature layer of biofilm with corncobs,

Scanning electron microscopy produces images with grey scales. The human vision can differentiate about 30 shades of grey in a monochrome image wheresas, hundreds of different colors can be distinguished. Peudocoloring is a technique artificial colors are applied to s grey

scaled image for better visualization. Here, asset of the images was manually pseudo-colored using Photoshop (Adobe Photoshop Version 22.4.2) outlining different morphotypes.

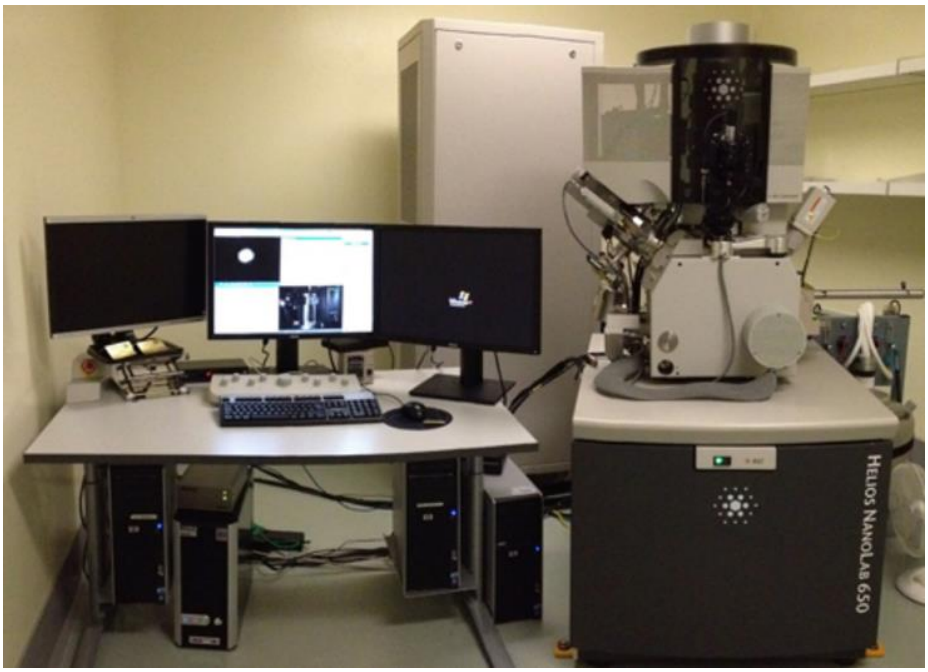


Figure 1. The Helios NanoLab 650 dual beam at the CHTP, University of British Columbia

4.3 Analysis of data

The biofilm images were captured at different levels in relation to the apico-coronal location of the bone loss on each specimen. The level of bone loss was determined from the radiograph using the most advanced mesial or distal site. Each implant fixture was imaged at three levels of bone loss (coronal, central and apical) and from two opposite sides (Fig. 3). At each location, three random 5000x images were taken, resulting in total of 18 images from each implant (3 levels x 2 sides x 3 images = 18 images).

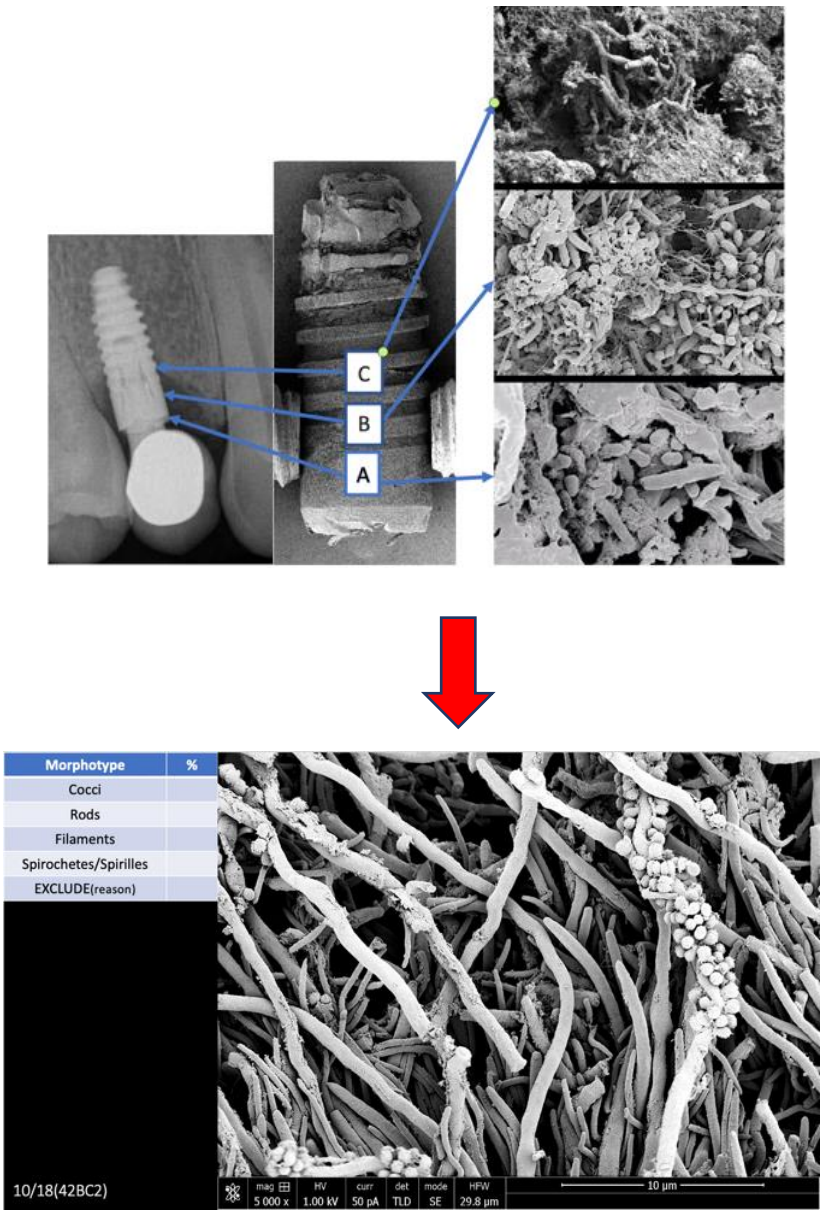


Figure 2. Representative illustration describing how the bacterial morphotypes were analyzed on failed implant surfaces. Images were randomly taken at three levels and opposite sites and the 5000x images were scored examiners for the morphotypes.

The most fundamental independent variables were the bacterial morphotypes and used to differentiate the morphotypes on the biofilm images. Three examiners received the images for determination of visual estimates of percentages of four main bacterial morphotypes. To assess the

inter-rater reliability, the first three samples were scored by the three investigators and the inter-rater reliability test was performed using Cohen's kappa testing in the IBM SPSS statistics software for Mac (SPSS Inc., Chicago, IL, USA). With kappa value >0.6, it was considered to be substantial to perfect agreement to proceed with the rest of the samples that were scored by all three examiners.[111] The scores for each image was averaged to one number (%) for each of the morphotypes. Therefore, at any level the six images received the mean number resulting in six replicate measurements for statistical purposes. The quantification data were compiled in Microsoft Excel (version 16.49) according to the examiners and samples. The statistical analyses included descriptive statistics (mean ± standard error of the mean) for each sample and the Mann-Whitney U test was used to compare two population means for independent variable of "Different surfaces [Ti-Unite (Nobel Biocare) vs. SLA (Straumann)]", "Mobility (reported clinical mobility or 100% bone loss)" and "Years in function [10 years and up vs. Less than 10 years]. The significance level was defined by $p \leq 0.05$. To compare the means difference with statistical significance, one-way ANOVA were performed. Additionally, post-hoc test, Tukey's test was performed to achieve the pair-wise comparision between all subgroups

Chapter 5: Results

5.1 Clinical and radiographical findings

A total of 14 failed implants were analyzed for the study (Table 1). The average age of the patient population was 67 years and there were 4 male and 10 female patients. The mean time in function for the implants was 7.9 years with range of 2 to 15 years. The majority of these implants had anodized surface (N= 11, Nobel Biocare). The amount of bone loss varied between 47.1 and 100% and five implants were mobile at the time of removal (Table 1).

Table 1. Demographic/Clinical information of specimens
(F=Female, M=Male; NB= Nobel Biocare and ST= Straumann)

Sample ID	Age (years)	Sex	Years in function	Surface Brand	Bone loss (%)	Mobility
1	45	F	5	NB	69.6	No
2	65	F	10	ST	65.0	No
3	70	M	15	NB	60.8	No
4	62	F	5	NB	78.9	No
5	62	F	5	NB	71.7	No
6	73	F	2	NB	100	Yes
7	67	M	10	NB	100	Yes
8	76	F	12	NB	47.1	No
9	61	F	10	NB	62	No
10	65	F	7	NB	57.7	No
11	63	F	6	ST	91	No
12	67	F	2	NB	100	Yes
13	87	M	10	NB	100	Yes
14	75	M	12	ST	100	Yes
Mean	67	M=4 F=10	7.9	ST:3 NB:11	80.4	Yes:5 No:9

Some of the images were excluded if the images were not quantifiable due to poor processing, poor image quality or lack of biofilm (Table 2). The overall exclusion rate of total images was 9%.

Table 2. Image information from each sample

Total images: 213, Excluded image: 21 (9.9%)

Sample ID	Surfaces	Total number of images	Excluded images
1	2	16	2
2	2	18	0
3	2	18	0
4	1	9	0
5	1	9	0
6	2	15	3
7	2	15	3
8	2	15	3
9	2	15	3
10	2	14	4
11	2	16	2
12	2	17	1
13	2	18	0
14	2	18	0
Total	26	213	21

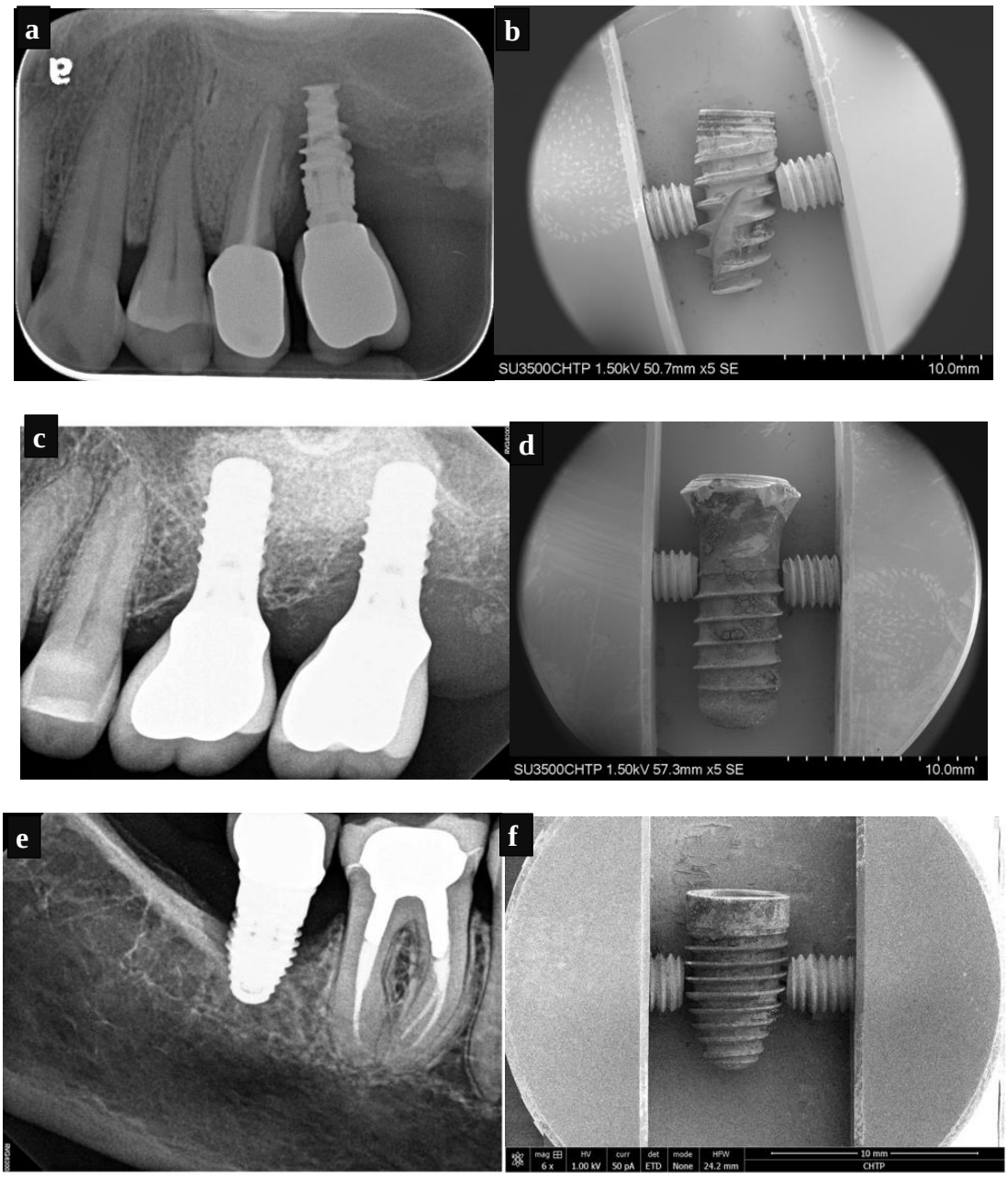


Figure 3. Radiographic images of a set of removed implants due to severe bone loss and how they were embedded for the imaging at SEM. Samples included mixture of Nobel Biocare implants (a-b,e-f) and Straumann implants(c-d) in various locations.

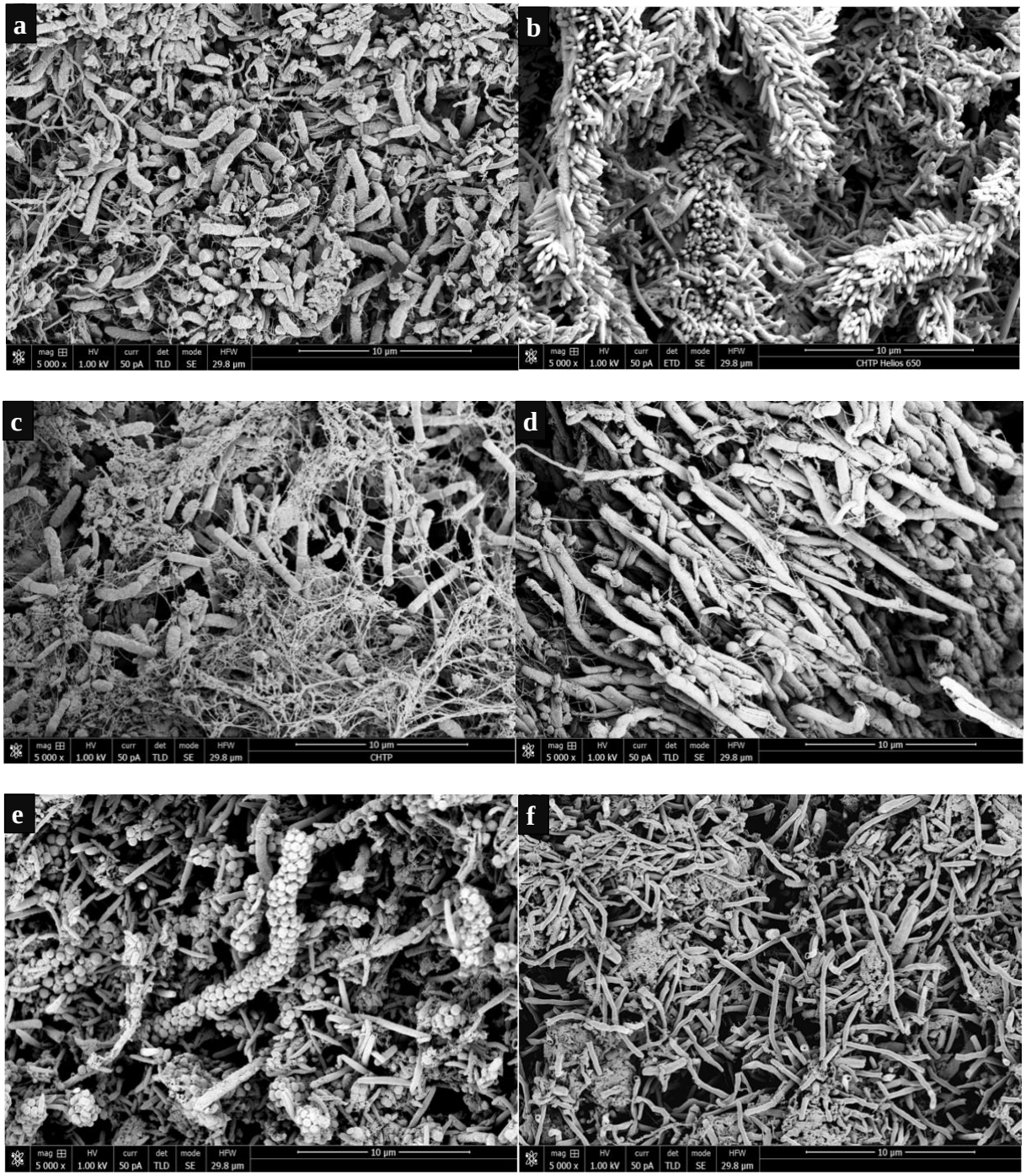


Figure 4. Example of images used for the quantification analysis at 5000X magnification. a-b (from the coronal third), c-d (from the middle third) and e-f (from the apical third) of the same implant.

5.2 Association of morphotypes to clinical findings

Table 3. Cohen's Kappa-values

	No. of images	Examiners 1 vs. 2	Examiners 1 vs 3	Examiners 2 vs 3
Sample 1	14	0.76	0.77	1.00
Sample 2	18	0.74	0.66	0.62
Sample 3	18	0.83	0.78	0.63

Once the inter-rater reliability was confirmed to be good, the descriptive statistics were performed using the mean and standard error of mean on each sample.

Table 4. Quantification of morphotypes for each sample

(Cocci, Rods, Filaments, Spirilla/Spirochetes) at three different levels (coronal, middle and apical)

Sample	Location	Cocci(MEAN± SEM)	Rods(MEAN± SEM)	Filaments(MEAN± SEM)	Spiro/Spirilles(MEAN± SEM)
1	Coronal	4.17 ± 1.01	39.72±5.82	12.22±2.92	43.89±6.32
	Middle	6.47±2.38	68.82±5.28	8.82±2.56	15.88±3.46
	Apical	9.55±3.12	58.64±4.58	17.27±5.4	14.55±4.79
2	Coronal	5.00±1.66	23.82±6.41	14.71±3.67	56.47±7.51
	Middle	10.67±2.96	18.00±3.80	14.00±3.17	57.33±5.02
	Apical	12.50±3.96	41.00±3.56	15.00±4.35	31.50±3.88
3	Coronal	7.22±2.22	25.00±3.62	55.00±5.69	12.78±4.67
	Middle	7.50±2.03	33.06±3.62	53.89±3.69	5.56±1.56
	Apical	13.61±2.21	37.22±3.44	45.56±3.98	3.61±1.56
4	Coronal	33.89±9.71	34.44±9.15	31.67±2.36	0.00±0.00
	Middle	33.33±8.50	17.78±4.94	40.00±13.84	8.89±1.62
	Apical	65.00±1.67	18.33±2.76	15.00±2.50	1.67±1.18
5	Coronal	0.00±0.00	65.56±14.82	33.33±14.34	1.11±1.11
	Middle	21.67±7.17	61.67±10.87	16.67±7.64	0.00±0.00
	Apical	12.86±5.10	78.57±4.59	8.57±2.83	0.00±0.00
6	Coronal	0.83±0.61	50.78±8.51	31.17±5.53	17.22±4.36
	Middle	33.33±8.50	25.56±12.03	9.44±3.38	31.67±12.02
	Apical	71.67±2.94	19.17±2.69	6.00±1.49	3.17±.99
7	Coronal	62.67±5.78	13.28±3.57	24.06±5.79	0.00±0.00
	Middle	58.24±8.05	23.71±5.28	15.71±3.29	2.35±1.36
	Apical	65.56±4.44	24.22±4.57	10.22±1.60	0.00±0.00
8	Coronal	6.47±1.91	67.35±7.67	24.71±8.08	1.47±0.94
	Middle	5.56±1.56	51.39±9.30	41.11±10.18	1.94±.82
	Apical	1.67±1.18	12.22±3.64	82.22±4.87	3.89±1.39
9	Coronal	8.33±2.56	55.83±6.93	26.94±7.36	8.89±2.31
	Middle	13.82±2.66	59.71±4.19	13.82±5.60	12.65±2.75
	Apical	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
10	Coronal	3.89±1.25	22.22±7.49	71.39±8.41	2.50±1.09
	Middle	31.82±9.40	31.36±7.69	21.36±2.14	15.45±6.89
	Apical	27.08±10.01	46.67±8.10	18.33±2.84	7.92±1.79
11	Coronal	1.11±.76	33.06±8.16	63.61±8.20	2.22±1.73
	Middle	1.76±.95	35.00±6.61	58.24±5.83	5.00±1.55
	Apical	5.83±1.93	42.08±6.26	30.00±5.22	22.08±4.50
12	Coronal	5.00±1.95	84.58±3.56	10.42±2.92	0.00±0.00
	Middle	0.00±0.00	2.94±1.66	79.41±7.74	17.65±7.45
	Apical	0.00±0.00	0.00±0.00	75.56±9.37	24.44±9.37
13	Coronal	44.33±9.30	12.22±4.54	43.44±7.18	0.00±0.00
	Middle	1.11±1.11	41.11±6.52	44.44±7.33	13.33±6.25
	Apical	3.82±2.56	36.76±5.30	55.88±7.07	3.53±3.53
14	Coronal	3.06±1.00	1.11±.65	95.83±1.09	0.00±0.00
	Middle	1.67±0.81	3.33±1.28	94.44±1.33	0.56±0.38
	Apical	1.94±0.92	3.61±1.13	93.33±1.57	1.18±0.81

Histograms depicting the relative distribution of morphotypes in each location of sample [coronal, middle, apical] of bone loss area in each sample are included in the appendix. The combined descriptive statistics are as follows.

Table 5. Distributions of the different bacterial morphotypes in different/combined locations in all samples

	Morphotypes			
	Cocci (MEAN± SEM)	**Rods** (MEAN± SEM)	**Filaments** (MEAN± SEM)	**Spirochete/Spirilla** (MEAN± SEM)
Coronal	13.28 ± 1.61	35.50±2.28	39.85 ±2.36	11.37 ±1.48
Middle	14.07 ± 1.57	33.87± 2.10	39.25± 2.47	12.81± 1.51
Apical	21.07 ± 2.2	28.98 ±1.92	40.86± 2.76	9.14 ±1.41
Combined	15.72 ± 1.03	33.12 ±1.25	39.92± 1.45	11.26 ±0.86

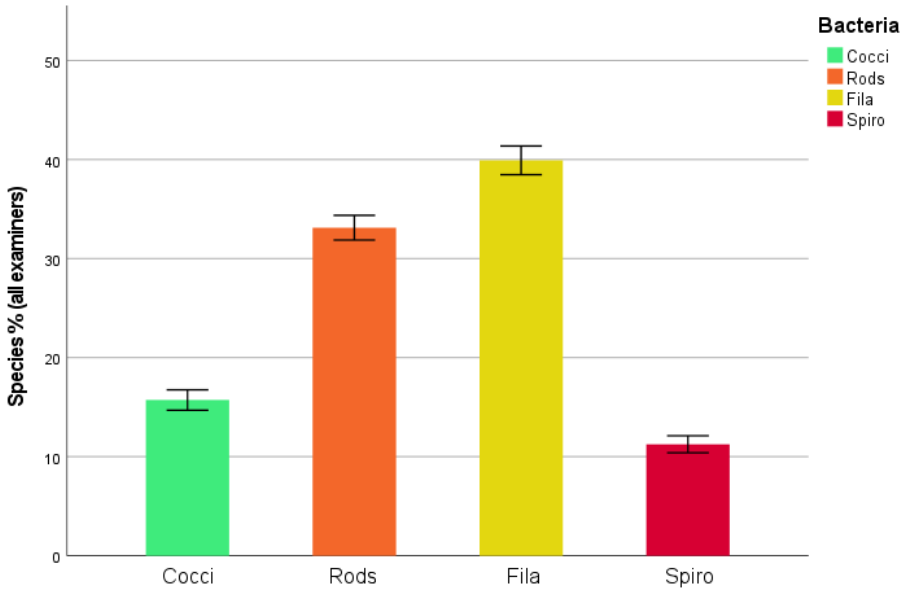


Figure 5. Relative distribution of each morphotype in different/combined locations in all samples

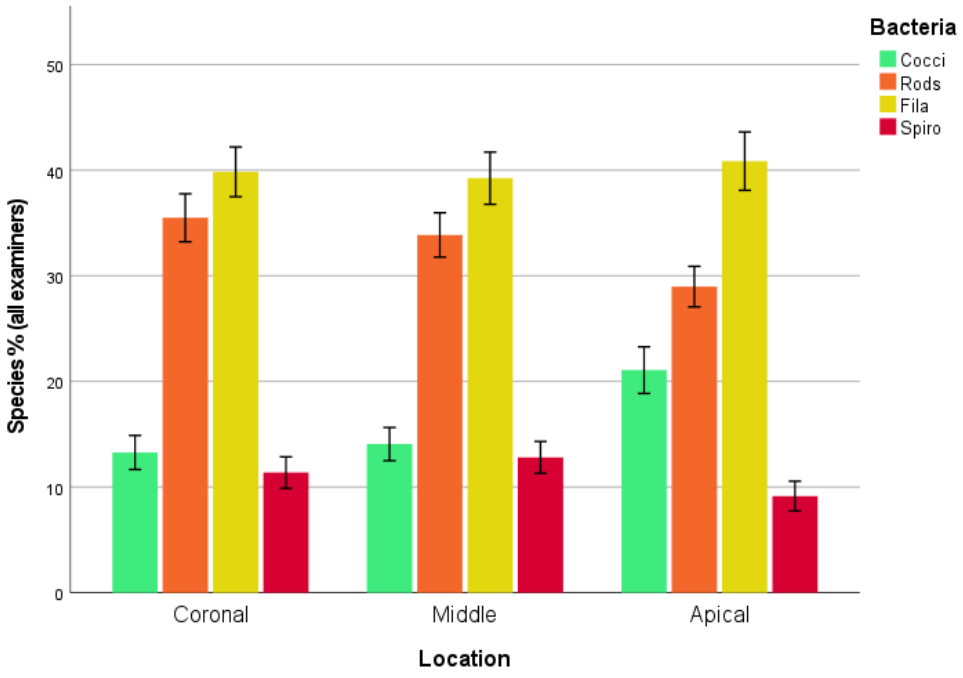


Figure 6. Relative distribution of each morphotype all combined samples in each location

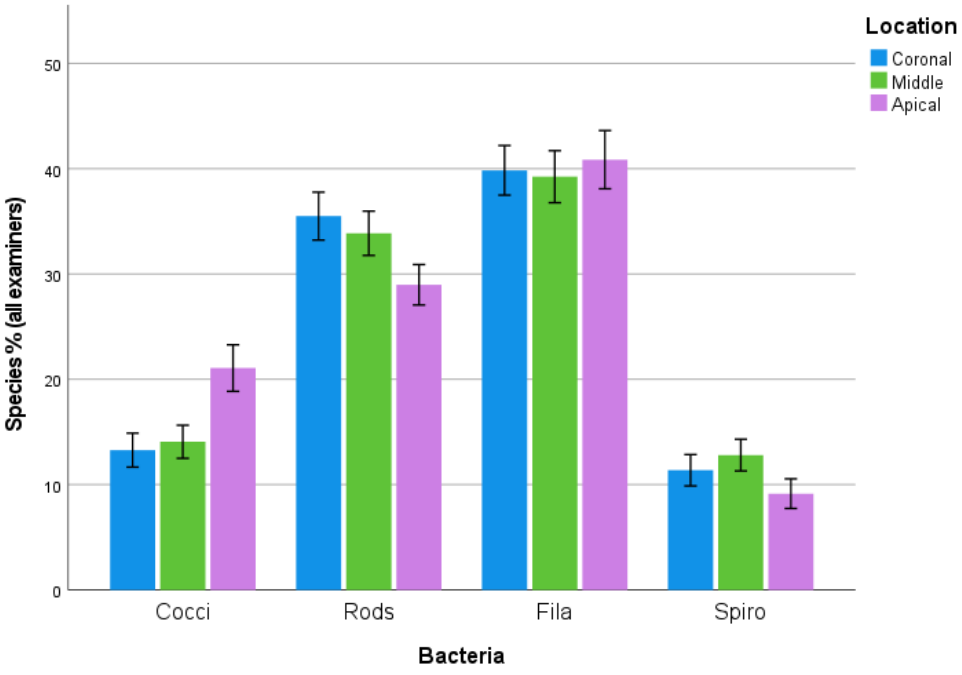


Figure 7. Relative distribution of morphotypes in each location of combined samples

5.3 Differences of morphotypes in different locations

The percentage distribution of bacterial morphotypes on different levels of failed implants is provided in Table 4. The results analyzed with One-way ANOVA showed that rods, filaments and sprilla/spirochetes morphotypes were not significantly different in the different locations. Overall rods and filaments dominated all the implant surfaces with combined load of 70-75% of all morphotypes, regardless of the location. Cocci (13-21%) and spirochetes/spirilla (9-13%) composed the rest of the morphotypes. Interestingly, cocci showed the highest proportion in the apical level (Table 4; Fig. 6 and 7). However, filament morphotype was the most dominant species in apical location (40.86 %). Overall and surprisingly, the variation of morphotypes at different levels of individual implants was low i.e. each implant possessed similar morphotypes throughout the exposed threads from coronal to apical locations. Only one implant (# 2) showed significant colonization with spirochetes/spirilla (32-57%). Overall, the colonization by spirochetes/spirilla was relatively low through the specimen population (Table 4).

Table 6. One-way ANOVA comparison between morphotypes for each location

Location	Morphotypes	Mean (SE)	ANOVA overall	Post-hoc (Tukey's) test
Coronal	Cocci Rods Filaments Sprilla/Spirochetes	13.28^{A} (1.61) 35.50^{B} (2.28) 39.85^{B} (2.36) 11.37^{A} (1.49)	$F(3, 900) = 56.09$ $p < .001$	Cocci vs Rods $p < .001$ Cocci vs Fila $p < .001$ Cocci vs Spiro $p = .903$ Rods vs Fila $p = .402$ Rods vs Spiro $p < .001$ Fila vs Spiro $p < .001$
Middle	Cocci Rods Filaments Sprilla/Spirochetes	14.07^{A} (1.57) 33.87^{B} (2.10) 39.25^{B} (2.47) 12.81^{A} (1.51)	$F(3, 836) = 48.04$ $p < .001$	Cocci vs Rods $p < .001$ Cocci vs Fila $p < .001$ Cocci vs Spiro $p = .968$ Rods vs Fila $p = .210$ Rods vs Spiro $p < .001$ Fila vs Spiro $p < .001$

Apical	Cocci Rods Filaments Sprilla/Spirochetes	21.07[B] (2.20) 28.98[C] (1.92) 40.86[D] (2.76) 9.14[A] (1.41)	F(3, 667) = 39.11 p < .001	Cocci vs Rods p = .044 Cocci vs Fila p < .001 Cocci vs Spiro p < .001 Rods vs Fila p = .001 Rods vs Spiro p < .001 Fila vs Spiro p < .001

Note: [A, B, C, D] different letters indicate significant difference between morphotypes (p < .05)

Table 7. One-way ANOVA comparison between locations for each morphotype

Morphotypes	Locations	Mean (SE)	ANOVA overall	Post-hoc (Tukey's) test
Cocci	Coronal Middle Apical	13.28[A] (1.61) 14.07[A] (1.57) 21.07[B] (2.20)	F(2, 601) = 5.37 p = .005	Coronal vs Middle p = .942 Coronal vs Apical p = .007 Middle vs Apical p = .019
Rods	Coronal Middle Apical	35.50[A] (2.28) 33.87[A] (2.10) 28.98[A] (1.92)	F(2, 601) = 2.29 p = .102	Coronal vs Middle p = .843 Coronal vs Apical p = .092 Middle vs Apical p = .270
Filaments	Coronal Middle Apical	39.85[A] (2.36) 39.25[A] (2.47) 40.86[B] (2.76)	F(2, 601) = 0.10 p = .908	Coronal vs Middle p = .983 Coronal vs Apical p = .958 Middle vs Apical p = .900
Spirilla/ Spirochetes	Coronal Middle Apical	11.37[A] (1.49) 12.81[A] (1.51) 9.14[B] (1.41)	F(2, 601) = 1.41 p = .246	Coronal vs Middle p = .757 Coronal vs Apical p = .556 Middle vs Apical p = .216

Note: [A, B, C] different letters indicate significant difference between locations (p < .05)

5.4 Association of morphotypes with implants brands

There were three samples of Straumann implants with the sodium chloride-treated hydrophilic 'SLA'(Sand-blasted, Large-grit, Acid-etched surface) and 11 samples of Nobel Biocare implants with the electro-chemically oxidized 'TiUnite' surfaces. The average years in functions were 7.5

years and 9.3 years with average bone loss of 77 % and 85 %, for the TiUnite and SLA implants, respectively.

Table 8. Relative distribution of morphotypes in TiUnite vs. SLA surfaces

			Morphotype Mean(±SEM)			
Surface	**Years in Function**	**Average bone loss**	**Cocci P<0.05**	**Rods P<0.05**	**Filaments P<0.05**	**Spirochete/Spirilla P<0.05**
Ti-Unite	7.54	77.06 %	19.28(1.29)	37.05(1.45)	34.50(1.54)	9.18(0.87)
SLA	9.33	85.33 %	4.27(0.61)	20.45(2.08)	57.41(3.17)	17.99(2.44)

Note: p-values are reported for Mann-Whitney test, p<0.05 is significant, n(TiUnite)=11, n(SLA)=3

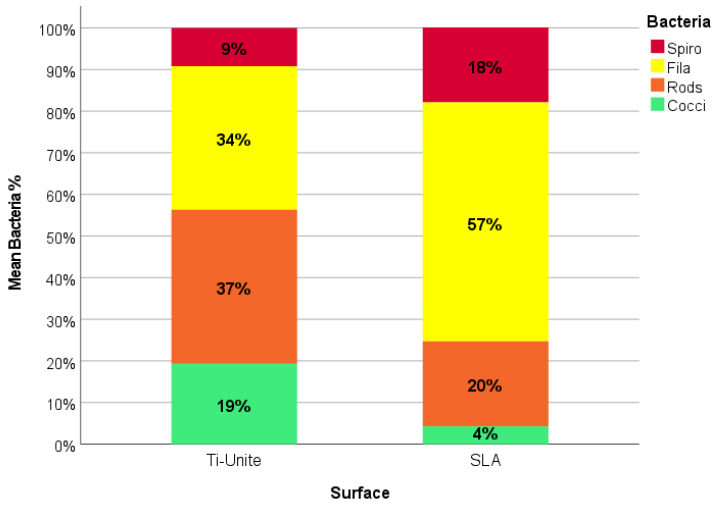


Figure 8. Relative distribution of morphotypes in TiUnite and SLA surfaces

There were significant differences in relative distribution of four different morphotypes in comparing two different kinds of surfaces. Most dominant species for both the 'TiUnite' and 'SLA' surfaces were rods and filaments, respectively. Cocci's relative distribution was higher in the TiUnite surface compared to SLA with 19.28% and 4.27% respectively. In addition, rods were more dominant on the TiUnite surface compared to the SLA surface with 37% and 20 %

respectively. However, there were more filaments and spirochetes on the SLA vs. Ti-Unite surface. This data need to be interpreted with caution as the TiUnite surfaces dominated the specimens by 11 to 3.

5.5 Association of morphotypes with mobility

The sample size for non-mobile implants group and mobile implants group were 9 and 5. The average years in function for the implants that were mobile at the time of explantation was 7.2 years and that of the non-mobile implant was 8.3 years. Average bone loss on the mobile implant group was 100% by definition and 67 % for those implants that were still firm at the time of explantation.

Table 9. Relative distribution of morphotypes in mobile vs. non-mobile samples

			Morphotype Mean (SEM)			
Mobility	**Years in Function**	**Average bone loss**	**Cocci** **P= 0.499**	**Rods** **P<0.05**	**Filaments** **P<0.05**	**Spirochete/Spirilla** **P<0.05**
Non-Mobile	8.33	67.09 %	11.41(0.93)	41.16(1.57)	33.50(1.65)	13.92(1.13)
Mobile	7.20	100 %	22.12(2.08)	21.17(1.79)	49.46(2.52)	7.28(1.28)

Note: p-values are reported for Mann-Whitney test, p<0.05 is significant, N(Non-mobile) =9, N(Mobile)=5

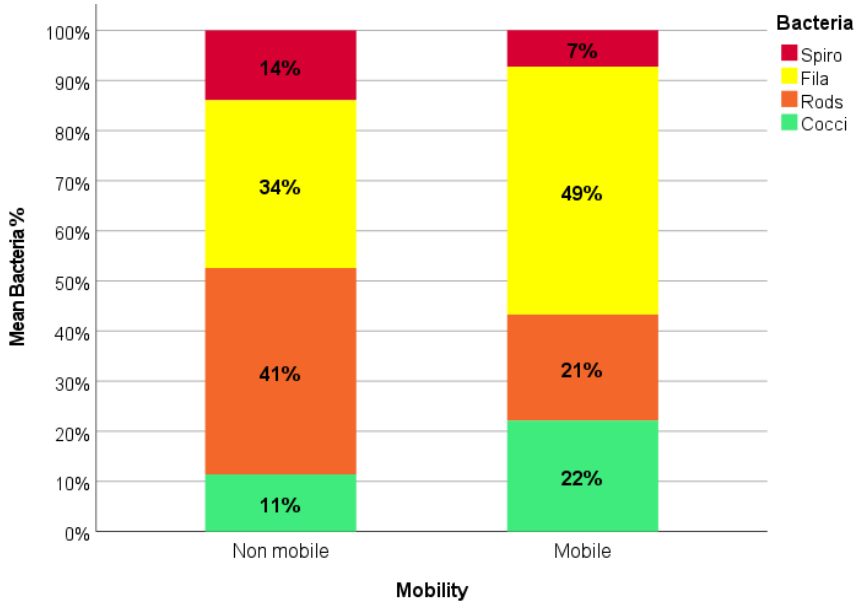


Figure 9. Relative distribution of morphotypes in mobile and non-mobile implants

These two different groups of implants had statistically significant differences in rods, filaments, and spirochetes/spirilla morphotypes. The dominant morphotypes of non-mobile implants were rods (41 vs 21%) while mobile implants were dominated by filaments (49% vs 34%; Table 9, Fig. 10). Rods were more abundant on non-mobile implant than mobile implants at 41% and 21%. Spirochetes/Spirilla were higher in the non-mobile implants over mobile implants at 14% and 7%, respectively.

5.6 Association of morphotypes with years in function of 10 year more or less

The sample size was seven implants in each group. The average years in function for the group of implants that had less than 10 years in function was 4.6. The other group of 10 and more years in function had an average year in the function of 11.3 years. Average bone loss on the ‘less-than-10 years’ and ’10 and more year groups were 81.3% and 76.4%, respectively.

Table 10. Relative distribution of morphotypes in failed implants relative to years in function (less than 10 years vs. 10 and more years).

			Morphotype Mean (SEM)			
Years in function	**Years in Function**	**Average bone loss**	**Cocci**	**Rods**	**Filaments**	**Spirochete/Spirilla**
			p=0.175	**P<0.05**	**P<0.05**	**P<0.05**
<10 years	4.57	81.27%	15.45(1.51)	37.46(1.98)	34.80(2.08)	12.29(1.29)
≥10 years	11.29	76.41%	15.95(1.40)	29.42(1.55)	44.02(1.98)	10.37(1.16)

Note: p-values are reported for Mann-Whitney test, p<0.05 is significant N (<10 years) =7, N (10 years and up) =7

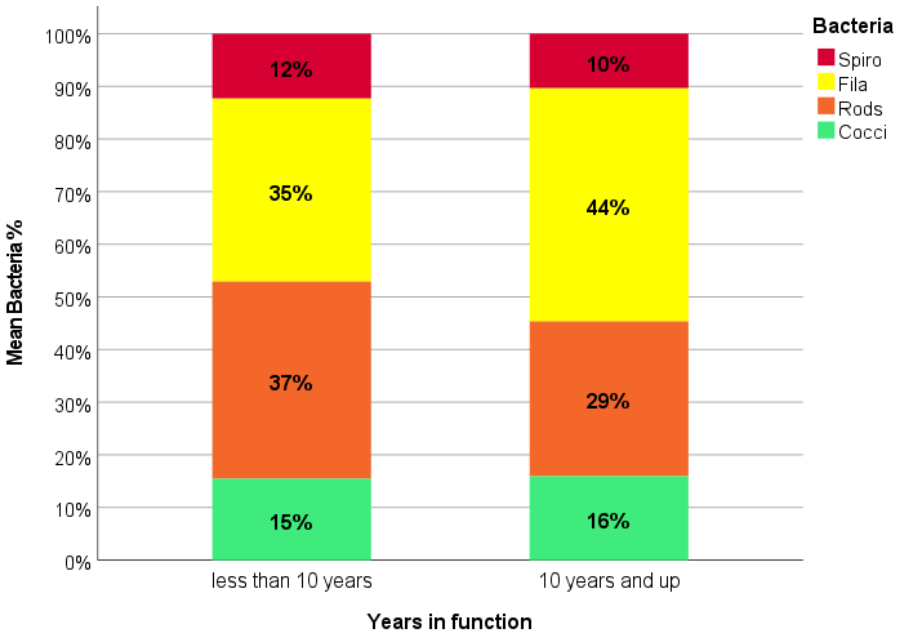


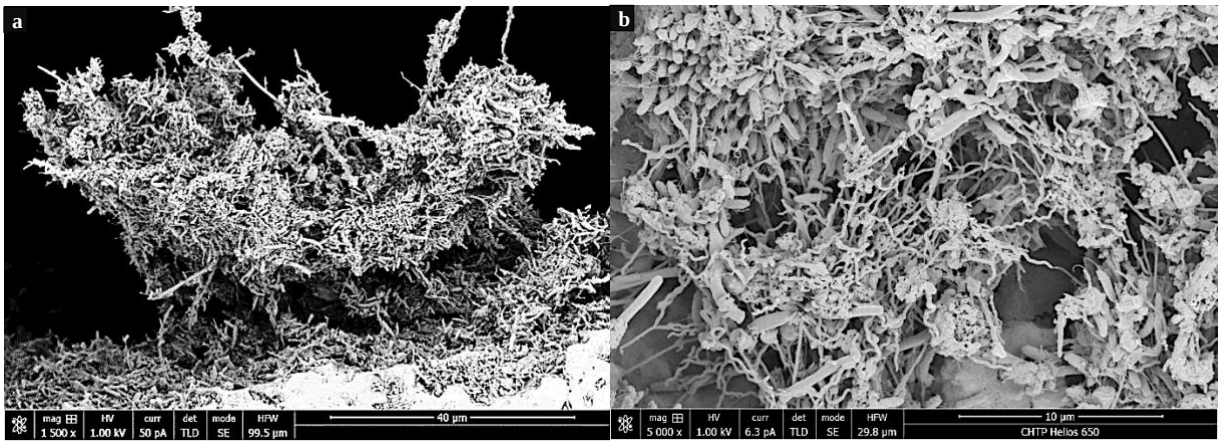
Figure 10. Relative distribution of morphotypes relative to years in function, <10 years vs. ≥10 years

Except for cocci, all morphotypes of bacteria had statistically significant differences in the relative distribution of morphotypes. The dominant morphotypes of the 'less-than-10 years' group were rods (37.5%) while filaments (44.0%) were the most prevalent morphotype for the '10 years and

more' group, respectively. The presence of spirochetes/spirilla were present in equal proportions in each group.

5.7 Images of biofilm on the surfaces of the failed implants

In this section, examples of individual biofilms present on failed dental implants are illustrated. Even though high-level heterogeneneity of biofilm with the various morphotypes of bacteria was common (Fig. 12a), rods and filaments were the dominant morphotype in all failed implants (Fig. 12b – d). The early stage of corncobs was captured with the filaments slowly being occupied by cocci (Fig. 12f)



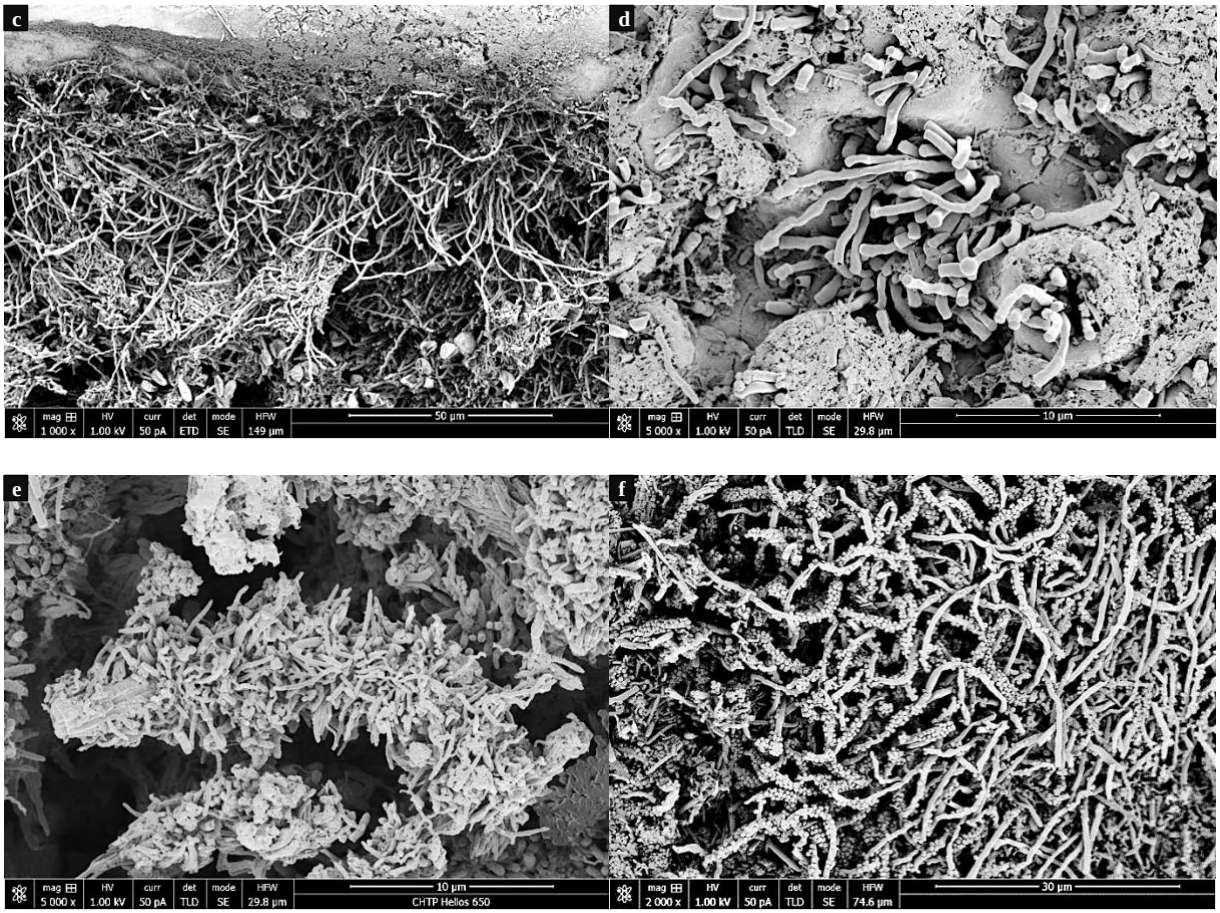


Figure 11. Examples of the biofilm captured on the surfaces of failed implants.

a. Striking appearance of biofilm with highly heterogeneous mixture of morphotypes (1500X). b. Biofilm on the surface of implants with highly diverse morphotypes (5000X) c. Filamentous morphotype bacteria attached to implant threads (1000X). d. Rods and filamentous morphotypes occupied the pores of the anodized implant surface (5000X) e. Wider form of the test-tube pattern is evident with filaments and rods attached onto the main core filament (5000X). f. Bacteria forming multiple strands of corncob formation (2000X)

In higher magnifications, the images showed more intimate contacts among various species (Fig. 13). These highly magnified images illustrated different level of heterogeneity with communication, formation, and diversity. There were abundant matrix layers around or on top of bacteria (Fig 13a, f) and complex structures were often associated between bacteria (Fig 13e).

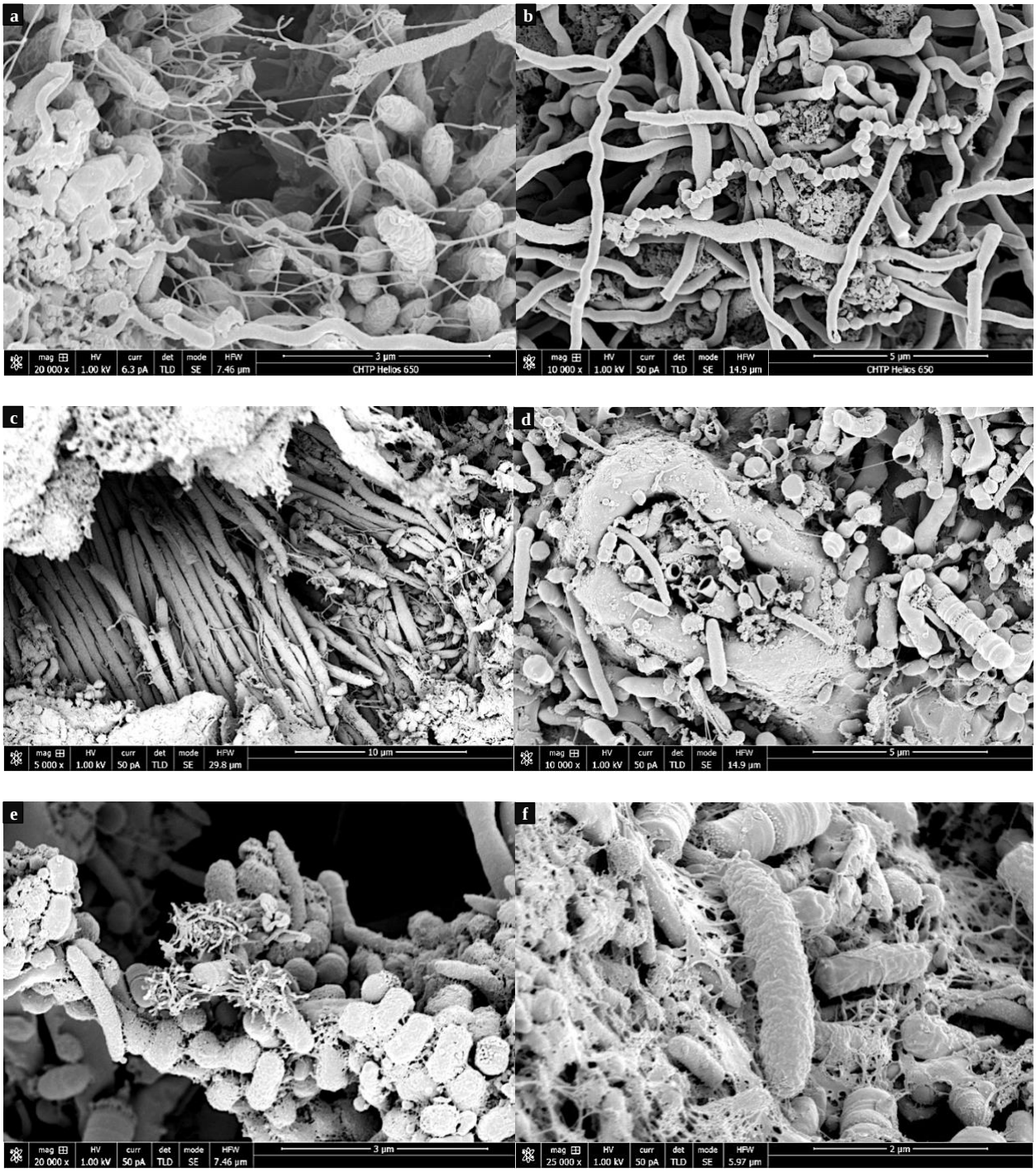


Figure 12. Examples of biofilm images in high magnifications on the surfaces of failed implants. a. Extracellular material interconnecting the multiple species (20000X) b. Cocci with filamentous species (10000X) c. Filamentous morphotypes arranged in palisading pattern (5000X) d. Various morphotypes, including cocci, rods, filaments, and small spirochetes gathering around the pores(10000X) e. Aggregates of rods and filaments (20000X) f. Various sizes and shapes of rod-morphotypes bacteria: round-ended rod in the centre, single and diplobacilli morphotypes adjacent to it (25000X)

Co-aggregation of bacterial species were often present, representing typical corncobs (Fig. 14a-b) and test tube-brush (14c-d) formations with central filament covered either by cocci or rods, respectively. Hedgehog formation was evident but was more scarce compared to corncobs and test tube brushes complexes (Fig 14e-f).

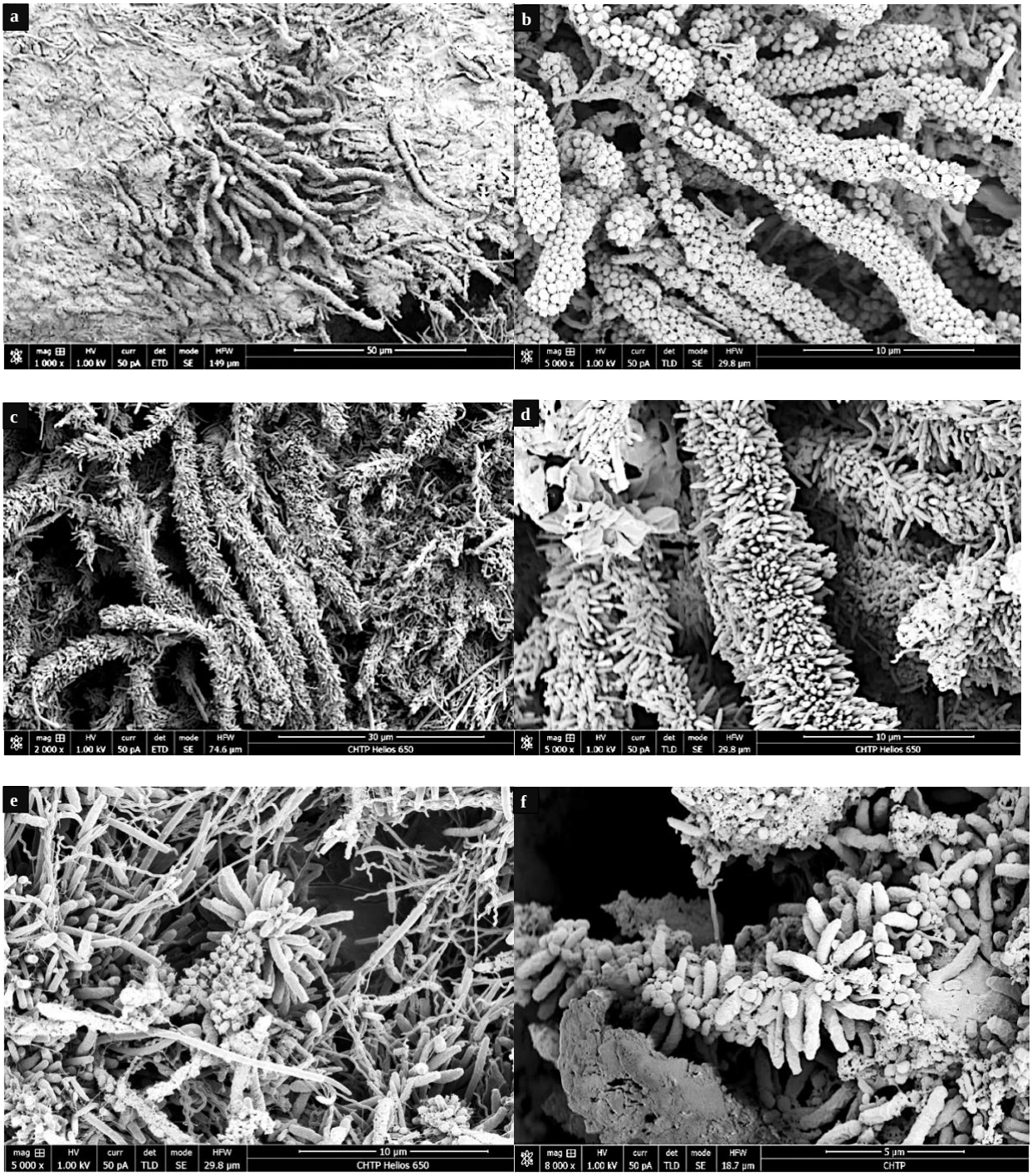


Figure 13. Examples of co-aggregate structures of the biofilm on the surfaces of failed implants

a-b. Corncobs in the coronal third location (1000X,5000X); c-d.Test-tube brush formations (in the middle third area (2000X, 5000X). e-f. hedgehogs structures on the middle third area (5000X, 8000X).

A set of samples was also imaged in cross-sectional orientation utilizing the ‘focused-ion beam’. The field of multiple corncobs was chosen and images captured in cross-sectional views (Fig. 15). These images illustrated the inner aspect of the corncobs layer. Even though it is hard to distinguish the morphotypes, relatively homogenous and dominant types of either rods or filaments were evident. Hollow spaces were most likely spaces occupied by the extracellular polymeric substances (Fig 15, c-d)

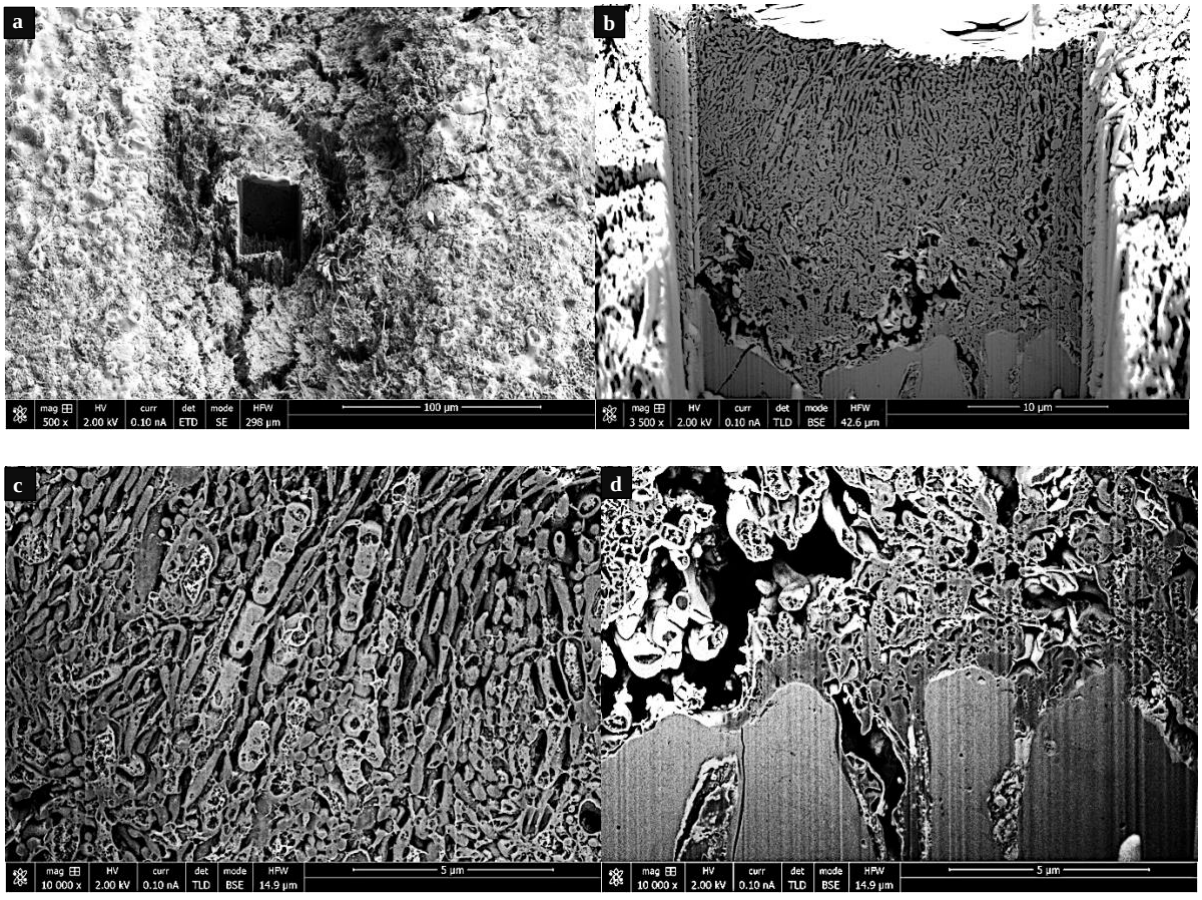
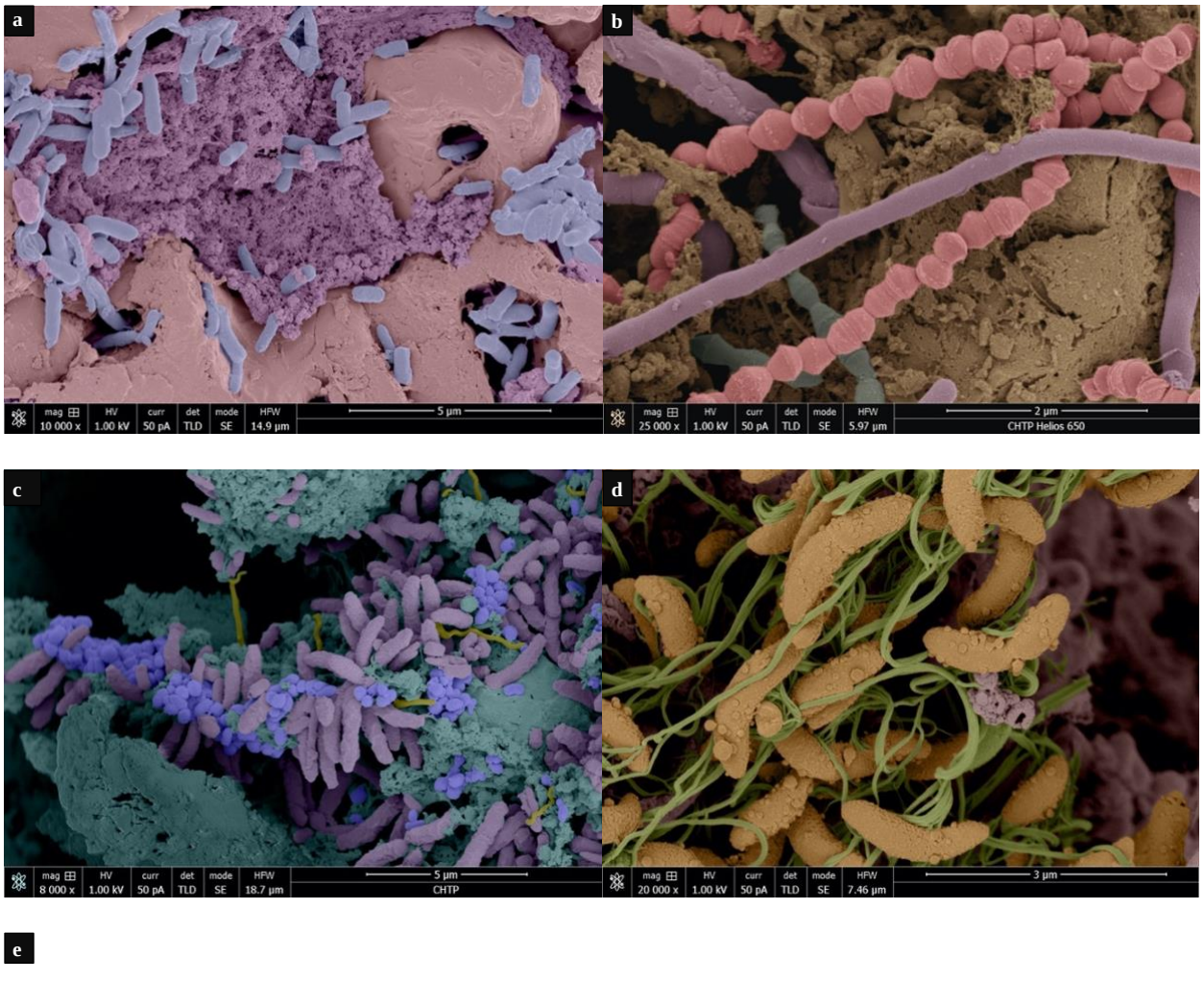


Figure 14. Examples of cross-sectional images of bacterial biofilm on failed implant
a. Area of corncobs was present on the top of the sample (Ti-Unite surface, 500X) b. Higher magnification of the same site illustrating densely packed bacteria dominated by filaments species (3500X) c. Cross sectional views showing filaments, rods and hollow spaces inside of bacteria (10,000X) d. Implant-biofilm interface illustraiing intimate contacts between biofilm and implant surface (10000X)

A set of biofilm images were pseudo-colored for a better visual examination. Using the images captured with high magnifications (usually more than 10,000X), different species and contrasing backgrounds and medium were colored for better visualization.

Some of rods were identified easily in relatively bare surfaces of implants with minimal amount of matrix (Fig 16a). Also utilizing different color schemes, it was easier to detect the coccus-like morphotypes vs. filaments (Fig 16b). Early stage of corncob formation was illustrated utilizing two different colors (Fig 16e).



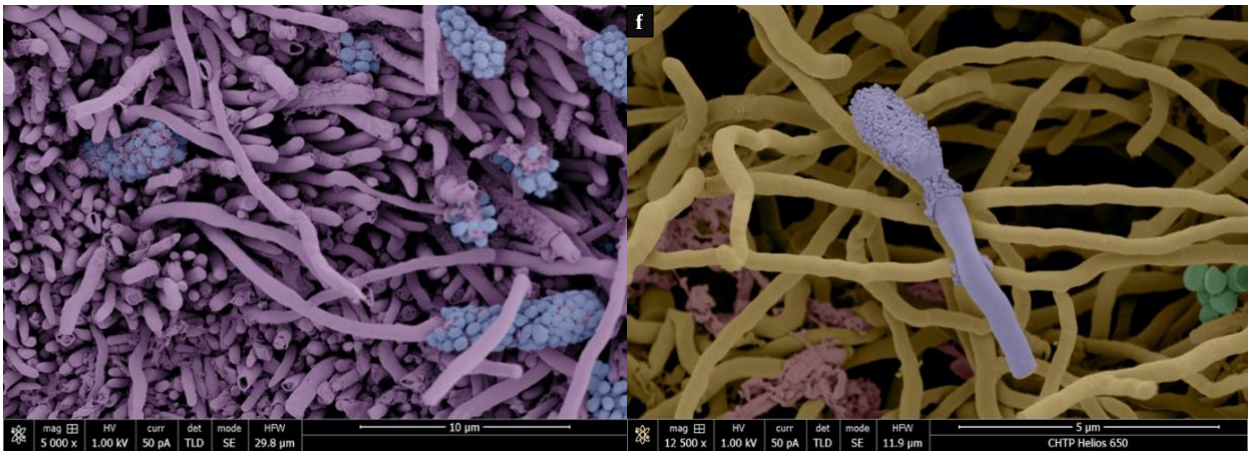


Figure 15. Pseudo-colored SEM images of the biofilm on the surfaces of the failed implant.
a. Rods (blue) around the pores of the anodized implant surfaces(10000K). The intercellular matrix is colored purple. b. Cocci (pink) along with filaments (purple) embedded in matrix (brown) (25000), c. Hedgehog formation with cocci (blue), rods (purple) around matrix (green) (8000X) d. Morphotype of vibrios (curved rods, brown) with elongated fimbriae (green)(20000K), e. Multiple strands of the corncob formation with filaments (purple) and cocci (blue) (5000X), f. Unknown morphotype (blue) with filamentous morphotypes (yellow), cocci (green) and matrix (brown)(12500X).

Chapter 6: Discussion

The main findings of the present study indicate that the biofilm covering failing dental implants is dominated by rods and filaments and lesser degree by cocci and spirochetes. In addition, many of the implants have the same morphotypes present throughout the implant surface. Furthermore, several failed implants contain biofilm that morphologically looks almost homogeneous mimicking infections by single organisms or a few species. The present study was not designed to explore the identity of the species. Nevertheless, these findings warrant discussion and reflections based on the known peri-implantitis microbiome research.

The resemblance between the microbiome between the teeth and implants has been described previosuly. Mombelli et al. (1987) were the first ones to report that the diseased implants with deep pocket more than 6 mm and suppuration sites had more prominent black-pigmented *Bacteroides*, *Fusobacterium spp.*, spirochetes, motile and curved rods[69]. This was confirmed by Sanz et al. (1990) that there was a resemblance between the periodontal disease and the peri-implantitis microbiomes[112]. Mombelli et al. (1987) also reported the comparison study between healthy and diseased implants using culturing and microscopic studies[69]. The diseased implants harbored more abundant gram-negative anaerobic motile species with rod morphotypes and fusiform species. However, there were no previous studies mapping the bacterial morphotypes on the surfaces of the biofilm on the clinically failed and removed implants to visualize the appearance of the community in situ.

Eick et al. (2016) investigated the difference of microbiota from the pockets around implants and adjacent teeth 10 years after implant placement[118]. The authors collected the plaque from the deepest areas of 504 implants and 493 adjacent teeth and compared them using DNA sequencing.

The authors reported that those known species of periodontitis were present in 6.2% to 78.4% of the implants depending on species. Especially four species that were more prominent on the implant surfaces were T. *forsythia,* P. *micra,* F. *nucleatum/necrophorum* and C. *rectus* when compared to adjacent teeth. Interestingly, the authors reported even though there were frequent presence of staphylococci, the *St. aureus* was scarce. The same group of authors previously reported high prevalence of *St. aureus* in peri-implantitis infection utilizing different molecular technology, checkboard technique[119]. They reported that *St. waneri* was most prevalent while *St. aureus* was rare. It is possible that the the study population and time in function may have contributed to the difference (1-year study (Salvi et al. 2008) vs 10-year retrospective study (Eick et al. (2015)). Time in function (less or more than 10 years) in our study was one of the independent factors in the present investigation showing a statistically significant change although the outcome did not differ (implant failure). Interestingly however, the filamentous morphotype increased and the spirochetes/spirilla morphotypes and rods morphotypes decreased while cocci morphotype did not change. The present study to characterize biofilm on failing dental implants has similarities to pioneering work by Listgarten et al. (1976) who described the different morphotypes in periodontitis lesions[4]. Subsequently, Listgarten and Hellden had quantified the microorganisms depending on their morphotypes in different locations[29] (see below).

The results showed there was no apparent transition from the coccoid- and rod-dominant morphotypes to more filaments and spirochetes/spirilles with the advancement of bone loss (from crestal to apical surfaces) on the failed implants. Therefore, the main hypothesis of the study was rejected and warrants further discussion.

6.1 Comparison to biofilm on the teeth

Ever since the importance of biofilm as a risk factor for the development of periodontitis, oral biofilm has been extensively studied and investigated[113]. There are more than 750 estimated species in an oral microbiota some of which are not even cultured as of today[114]. Listgarten et al. (1976) studied the bacterial flora on the extracted teeth and described the morphotypes of bacteria of various clinical stages such as health, gingivitis, periodontitis, periodontosis and post-periodontosis.[4] The distinct morphotypes were noted, including coccoid species in the healthy sites and an increased amount of filamentous types evident in the inflamed gingival tissues. Initially, the dominant species included Gram-positive cocci that were aligned perpendicular to the tooth surface. As time lapsed, the authors reported that the filaments were evident on the top of the layer of cocci. In three weeks, the filaments were so dominant and the cocci were scarce. After three weeks, there was no further morphological evolvement except the free-floating spirochetes layers evident on the most superficial layer. The authors reported the presence of corncobs-like structures as complexes consisted of filaments surrounded by cocci. Also, the corncobs were described being on the surface of this bacterial mass and speculated to represent a transition to anaerobic flora with spirochetes as the severity of disease worsened. The current investigation witnessed similar features in the peri-implantitis biofilm, including frequent corncob formations that sometimes dominated along the entire implant surface. However, spirochetes appeared to be less frequent than in periodontitis biofilm described by Listgarten. Corncobs are also a frequent finding in older supragingival plaque.

Only a few studies have identified different species of bacterial in native biofilm on teeth. Zijnge et al. (2010) showed the biofilm on the natural teeth and analyzed the architecture of the biofilm using Fluorescent In Situ Hybridization (FISH) technique[120]. The authors analysed ten (10)

teeth that were diagnosed with periodontitis from four (4) patients determined to be extracted. In subgingival biofilm investigation, the authors reported that there were four (4) different distinct layers depending on the bacterial morphotypes and intensities of fluorescence. The first layer showed only the *Actinomyces* sp. that have rod-shape as individual bacteria. The intermediate layer was reported to have *F. nucleatum (rod), T. forsythia (rod)* and *Tannerella* sp. (*rod*). Common species in the outer layers of biofilm and intermediate layers were '*Cytophaga-Flavobacterium-Bacteroides* cluster' as mixture of filamentous, rods-shaped or occasional cocci-shaped bacteria. Interestingly, the authors called the *Synergistetes* group A bacteria, a large 'cigar-like bacteria' in the superficial layer depicting them in a 'palisade' arrangement. A fourth layer was described as a loose layer with *Spirochetes* as a primary species. In these most superficial layers, the authors were able to detect the complexes of multi-species structures such as test-tube brushes. Ever since the test-tube and corncobs were described[4], this study identified the species associated with those complexes. The authors were able to identify those species forming the 'test-tube' brushes as *T. forsythia, Campylobacter sp., P. micra, Fusobacteria* and *Synergistetes* group. The core structures were consisted of *Tanerella forsythia, Fusobacterium nucleatum* and central axis of yeast cells or hyphae.The authors conclusion was congruent to the findings of Kolenbrander et al.(1993) that reported *Fusobacterium nucleatum* evident in the intermediate layer[117].

Mark Welch et al. (2016) reported the biogeographical analysis on supra- and subgingval plaque using the the same FISH technique. [115] In their analysis of multi-genus consortia, physical structures of filamentous species extending from the base and peripheral areas of extensions were coverd by cocci. In that context, these coaggregates appear to be a part of even larger superstructure, named hedgehog structures where *Corynebacterium* forms the foundation with long filaments

extending through the entire biofilm aggregate.[115] Towards the surface of this multigenus consortium, the tips of *Corynebacterium* filaments are coated by cocci or rods forming corncob-like structures. In that study, the corncobs had either single layer of *Streptococc*i or *Porphyromonas* covering the *Corynebacterium* filament or double layer consisting of a combination of *Streptococci* as the inner layer and *Heamophilus/Aggregatibacter* as the outer layer.[115] The most common corncob had single layer of *Streptoccci* and partial layer of *Heamophilus/Aggregatibacter*.[115] Previously, *F. nucleatum* has been shown to form corncobs *in vitro* but they were not commonly involved in these structures in supragingival plaque.[116,117] Nine taxa were common in forming the hedgehog complex: *Corynebacterium, Streptococcus, Prophyromonas, Haemophilus/Aggregatibacter, Neisseriaceae, Fusobacterium, Leptotrichia, Capnocytophaga, Actinomyces.* The authors reported it was *Corynebacterium* that forms the core filamentous axis and *Streptococcus* cells (*Hemophilus/Aggregatibacter*, *Porphyromonas*) were at the distal tip creating this unique spatial arrangement.[115]

The bacterial species forming the complex structures in biofilm associated with dental implants are likely similar to those observed on teeth. However, this needs to be confirmed in future studies. These type of studies (e.g. FISH) on metal surfaces may require development of new techniques that conserve the biofilm in situ as the metal implant can not be eliminated by decalcification.

Semeda-Pienaar et al. (2017) investigated the different bacterial morphotypes to be able to use to grade the severity of periodontal diseases such as periodontitis from gingivitis[113]. The authors' main focus was on the number of *Spirochetes* and its increase with statistical significance from health to periodontitis and the opposite pattern of the morphotypes of cocci and non-motile rods. However, the non-motile rods were shown an increasing pattern from health to gingivitis but their numbers were reduced in periodontitis. The authors reported the *Spirochetes* dominated in

periodontits (71%). The dominance of *Spirochetes* in periodontitis pockets have been demonstrated in multiple other studies as well.[29 121 122] The present study showed relatively low percentage of *Spirochetes* in biofilm attached to the titanium implants in peri-implantitis. This observation could be for multiple reasons. The pocket environment at the deepest sites of peri-implantitis has different immunological environment, including more acute phase cells and no separation of bacteria from inflammatory lesion by pocket epithelium. This difference to chronic periodontitis could perhaps lead to more efficient clearance of motile spirochetes. However, there could be multiple other reasons for underrepresentation of *Spirochetes*. *Spirochetes* are motile species and they maybe underpresented in the attached biofilm and present more abundantly in the unattached peri-implantitis pocket. Furthermore, the quantification method used in the present study could underestimate their relatively proportion as they are typically smaller in size than the other bacteria in the peri-implantitis biofilm.

There are several limitations of this study including the process of sample collection, fixation timing of samples, quantification of morphotypes and accuracy of determining the bone loss areas. The method of removing a failed implant may vary and damage the surface inevitably. Therefore, the biofilms analyzed for the present study may not have been 100% representative. There might have been contamination on the sample or translocation of the biofilm within the sample. Processing of the specimens may have also caused artefacts and removed some of the biofilm. Determining bone loss using the radiographs may not have been completely accurate depending on the quality of radiograph. Lastly, the quantification process might have been influenced by the three examiners. Those quantification numbers were individual ‘estimation’ rather than computed values. Therefore, individual bias could be present.

Chapter 7: Conclusion and Future Directions

7.1 Conclusion

Overall rods and filaments dominated all the implant surfaces with combined load of 70-75% of all morphotypes, regardless of the location. Each implant possessed similar morphotypes throughout the exposed threads from coronal to apical locations. The colonization by spirochetes/spirilla was relatively low through the specimen population.

There were significant differences in relative distribution of four different morphotypes in comparing two different kinds of surfaces. The dominant morphotypes of non-mobile implants were rods while mobile implants were dominated by filaments. Spirochetes/Spirilla were higher in the non-mobile implants over mobile implants. The dominant morphotypes of the ‘less-than-10 years’ group were rods while filaments were the most prevalent morphotype for the ’10 years and more’ group, respectively. The presence of spirochetes/spirilla were present in equal proportions in each group. Cross-sectional views of biofilm were illustrated to show the adundance of filamentous/rods morphotypes in the middle layer and intimate contacts between biofilm and implant surface. Pseudo-coloring techniques were applied for better visualization of diversity of microflora of the oral biofilm on the failed implants surfaces.

The current investigation showed high diversity in the biofilms on surfaces of failed implants due to peri-implantitis with similar clinical outcomes. Therfore, attempts for finding the pathogen cluster may be elusive.

7.2 Future Direction

The studies in regard to cleaning the implant surfaces due to the biofilm contamination is hot topic in the field implantology considering that the prevalence of peri-implantitis is high[123]. There has been a number of studies exploring different mechanical, chemical and physical methods to decontaminate infected implant surfaces.[124,125,126] It can be concluded that most studies report incomplete decontamination of the implant surfaces regardless of the method used. Only air flow with glycine seems effective in decontamination of SLA surfaces but its use is not recommended in open flap environment due to risk of emphysema.[127] Interestingly, Schuldt et al. (2021) recently published that platelets from autologous blood products such as leucocyte-platelet rich fibrin might serve as a promising new venue to decontaminate infected implant surfaces although its application in clinical setting could be cumbersome.[125]

The complex nature of native biofilm on implant surfaces on failed implants as observed in the present study presents a difficult task for decontamination and explains why peri-implantitis treatment regimens often fail or are only partially successful. More studies are needed that explore novel techniques on decontamination of native biofilms on failing implants.

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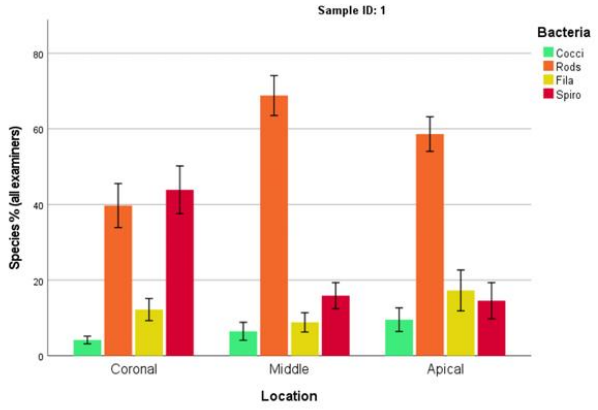
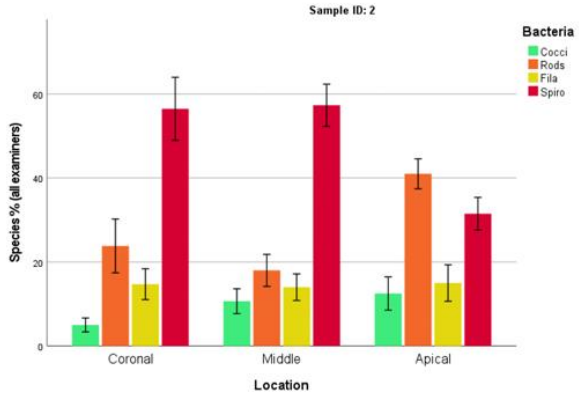
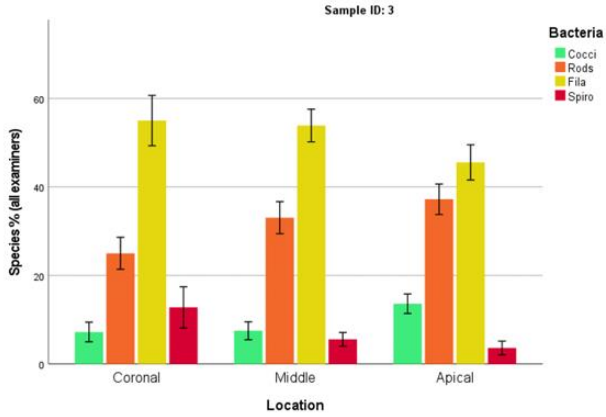
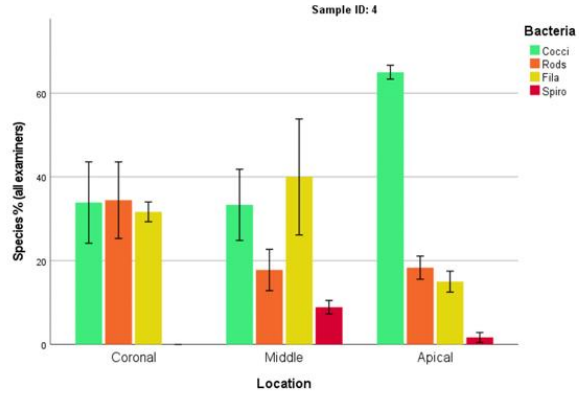
Appendix

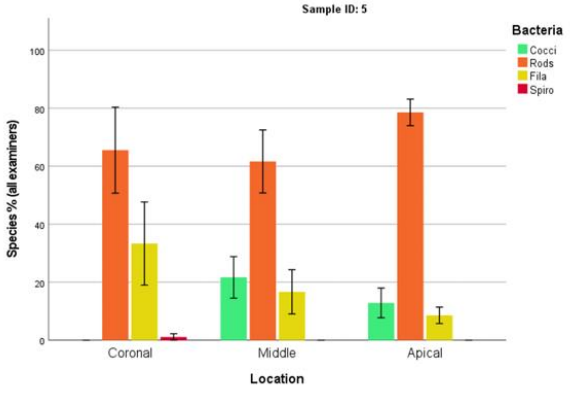
A.1 Histograms depicting the location distribution of each morphotype in each location

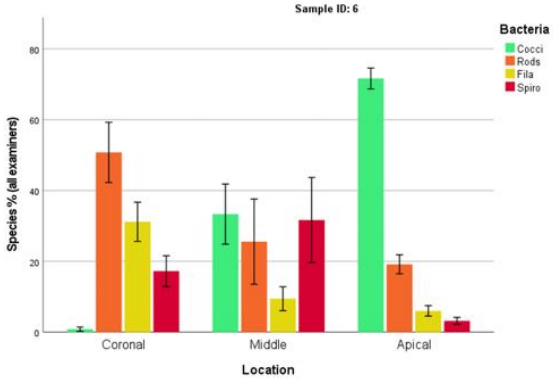


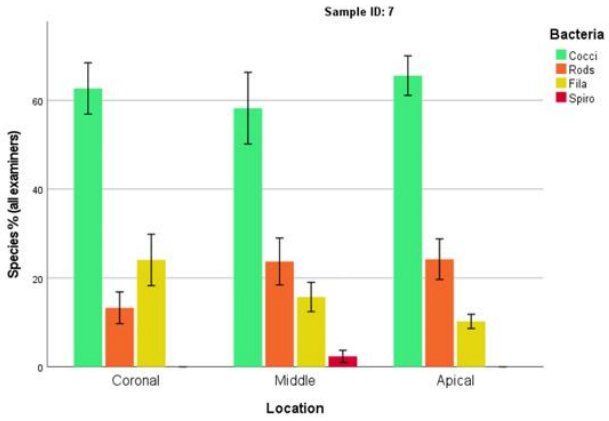


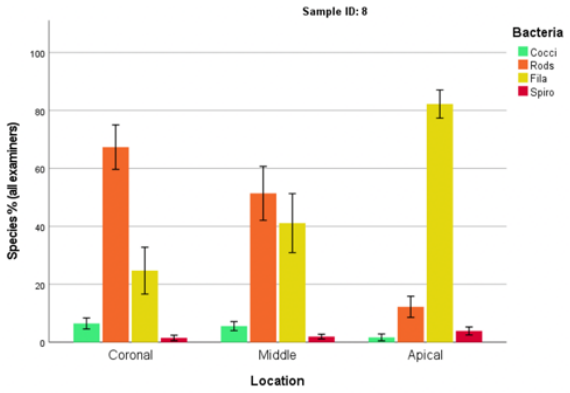









Sample ID: 7
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
20
40
60
Coronal
Middle
Apical
Location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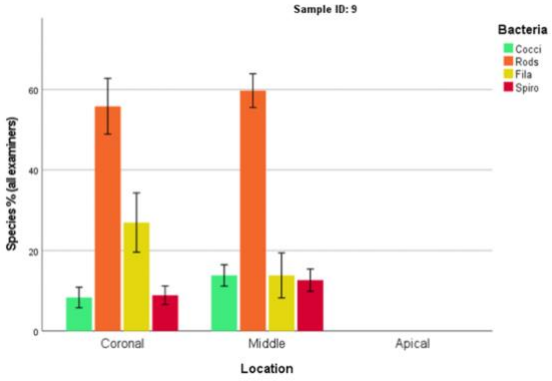
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Spiro
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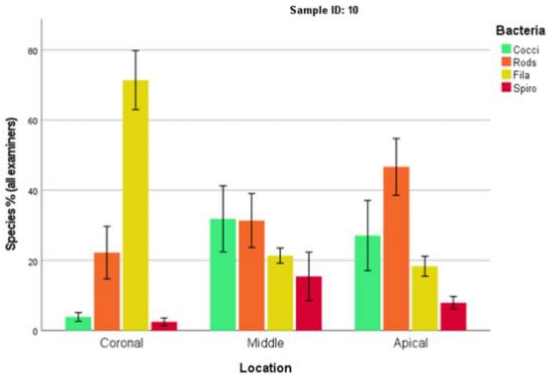
Coronal
Middle
Apical
Location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Rods
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Species % (all examiners)
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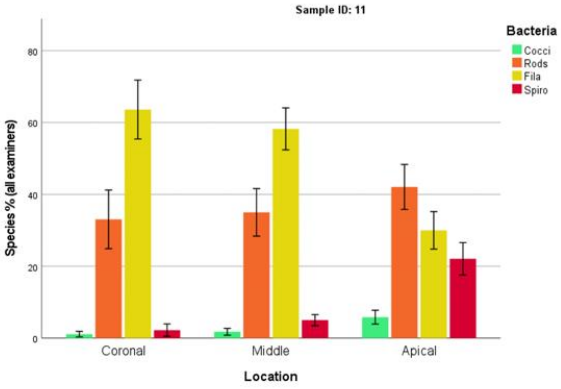
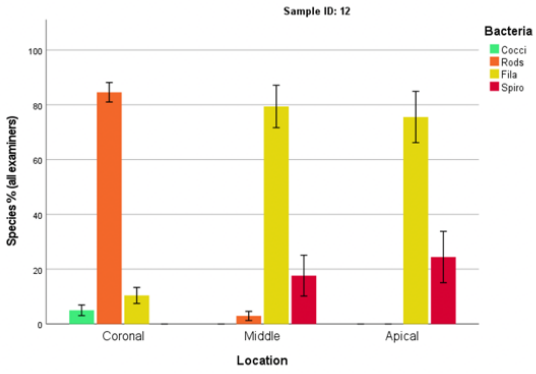
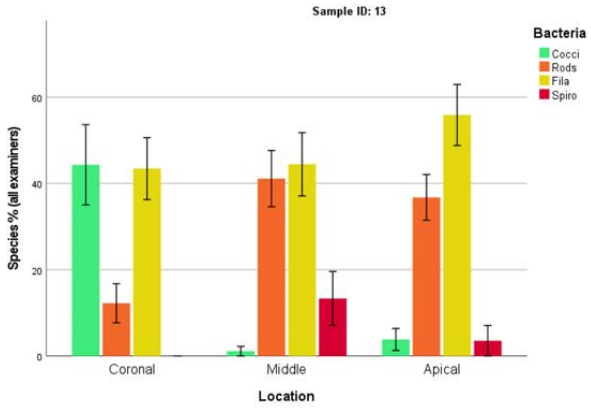
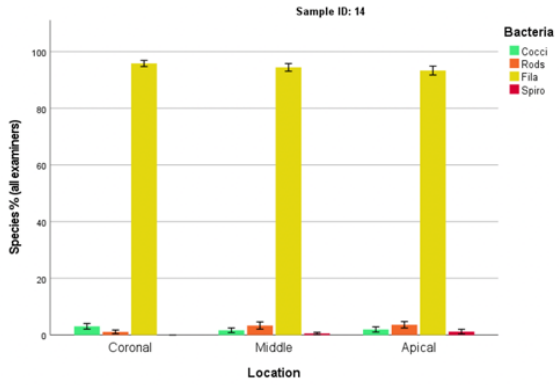
20
40
60
80
Coronal
Middle
Apical
Location

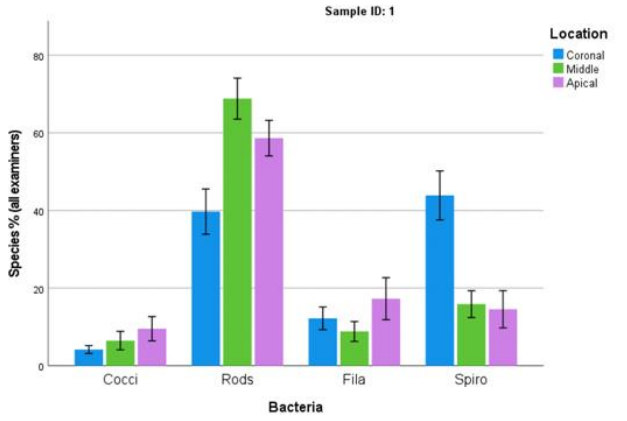

Sample ID: 11
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
20
40
60
80
Coronal
Middle
Apical
Location

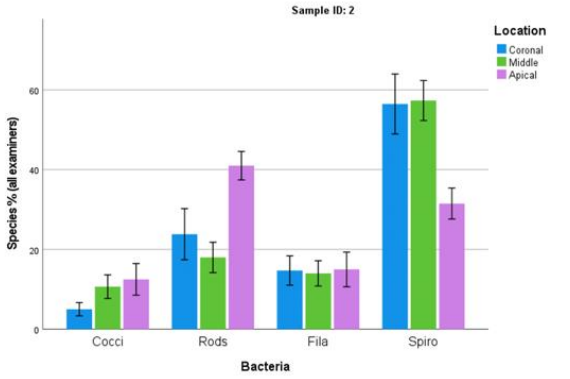

Sample ID: 12
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
20
40
60
80
100
Coronal
Middle
Apical
Location

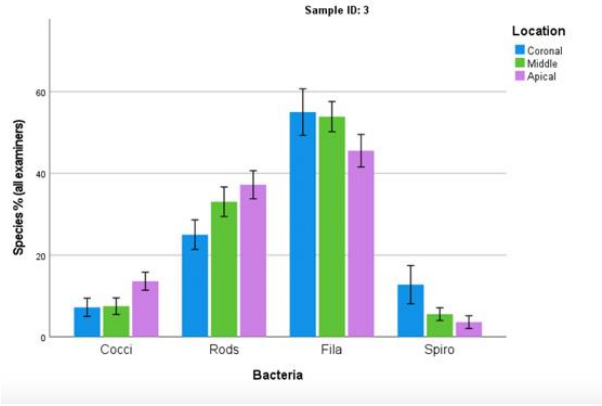

Sample ID: 13
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
20
40
60
Coronal
Middle
Apical
Location

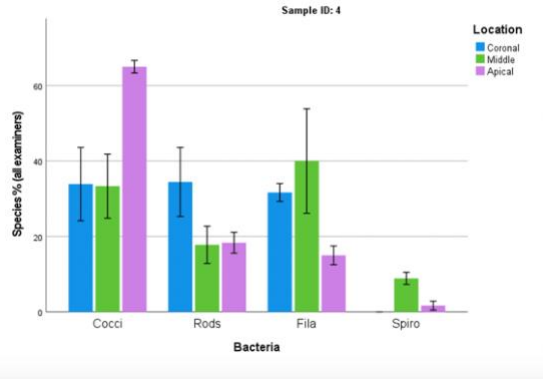

Sample ID: 14
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
20
40
60
80
100
Coronal
Middle
Apical
Location

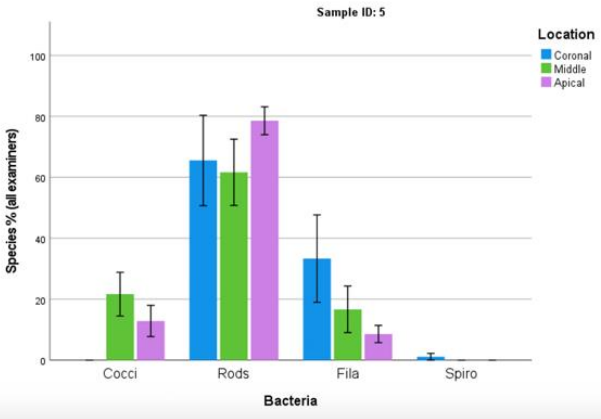
A.2 Histograms depicting the relative distribution of morphotypes of each sample

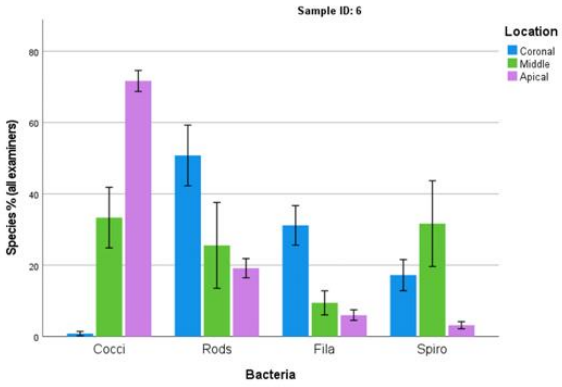


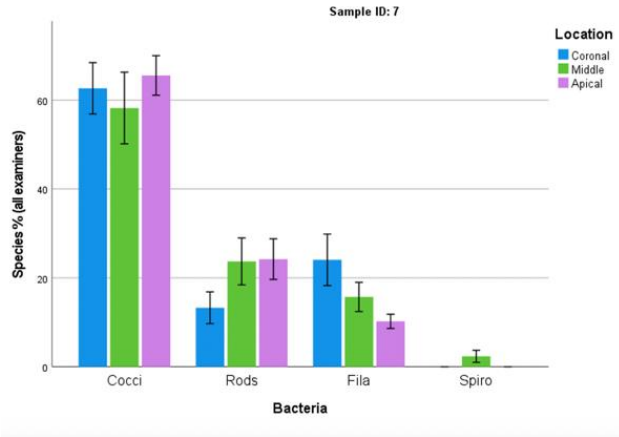


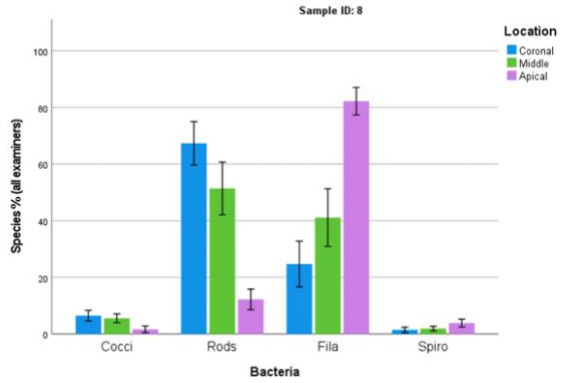


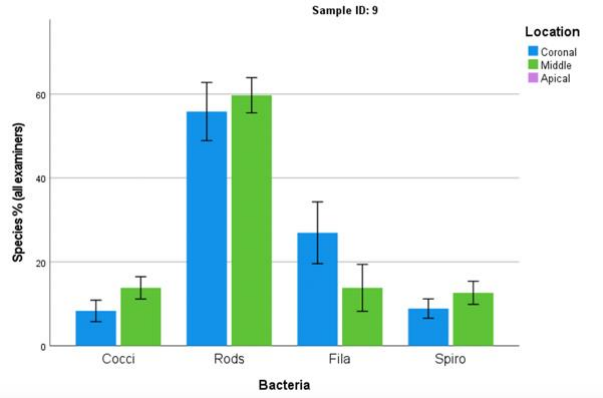


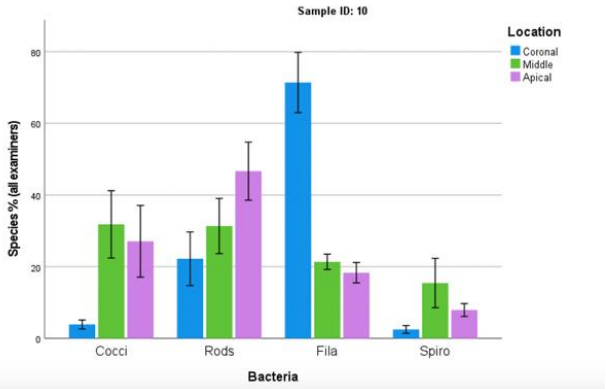


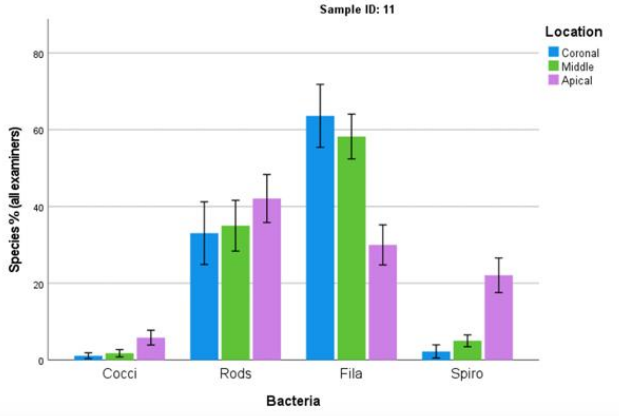


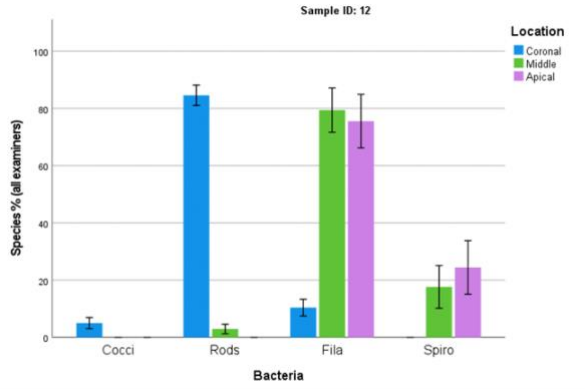

Sample ID: 7
Location
Coronal
Middle
Apical
Species % (all examiners)
0
20
40
60
Cocci
Rods
Fila
Spiro
Bacteria

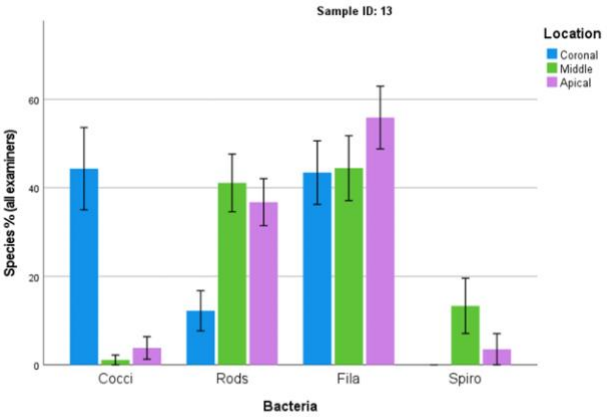

Sample ID: 8
Location
Coronal
Middle
Apical
Species % (all examiners)
0
20
40
60
80
100
Cocci
Rods
Fila
Spiro
Bacteria

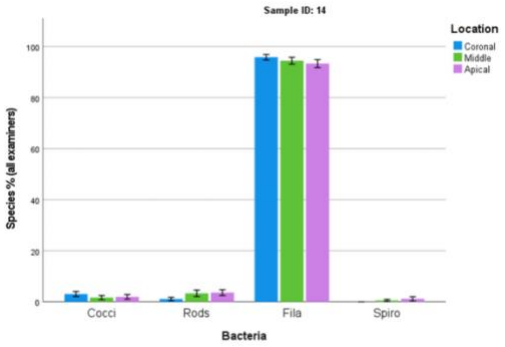

Sample ID: 9
Location
Coronal
Middle
Apical
Species % (all examiners)
0
20
40
60
Cocci
Rods
Fila
Spiro
Bacteria


Sample ID: 10
Location
Coronal
Middle
Apical
Species % (all examiners)
0
20
40
60
80
Cocci
Rods
Fila
Spiro
Bacteria

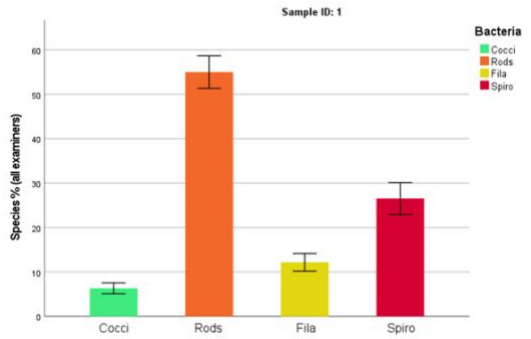

Sample ID: 11
Location
Coronal
Middle
Apical
Species % (all examiners)
0
20
40
60
80
Cocci
Rods
Fila
Spiro
Bacteria

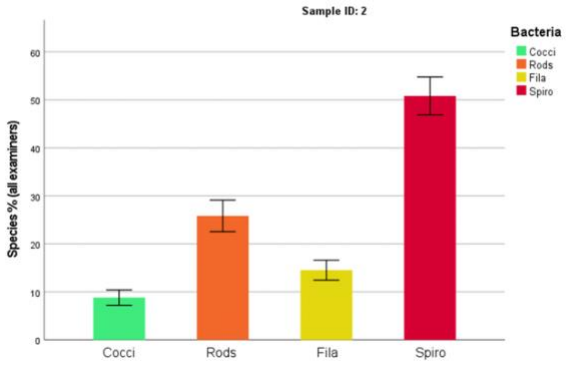

Sample ID: 12
Location
Coronal
Middle
Apical
Species % (all examiners)
0
20
40
60
80
100
Cocci
Rods
Fila
Spiro
Bacteria

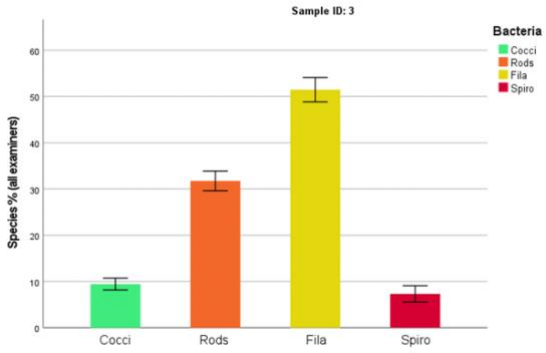

Sample ID: 13
Location
Coronal
Middle
Apical
Species % (all examiners)
0
20
40
60
Cocci
Rods
Fila
Spiro
Bacteria

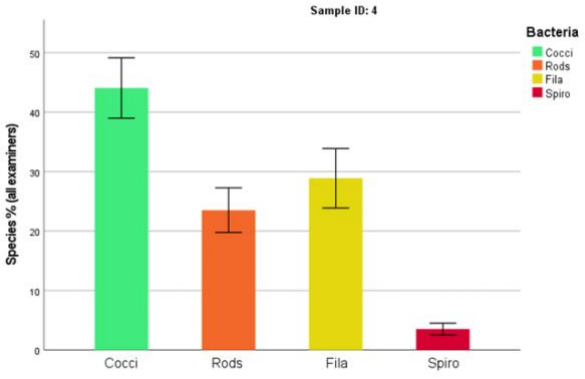

Sample ID: 14
Location
Coronal
Middle
Apical
Species % (all examiners)
0
20
40
60
80
100
Cocci
Rods
Fila
Spiro
Bacteria

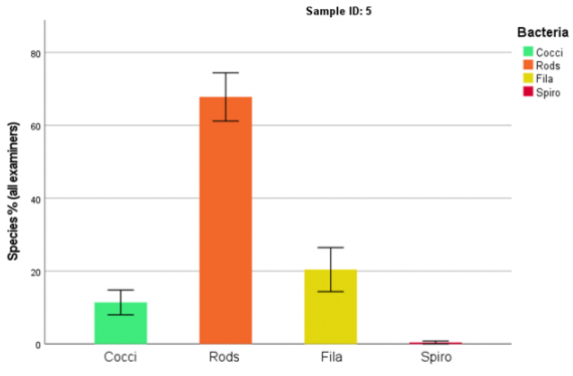
A.3 Histograms depicting the morphotypes in total in each sample

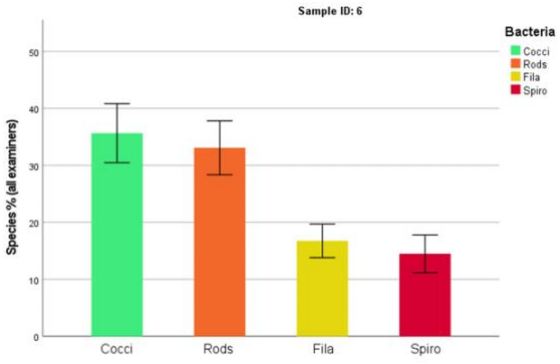


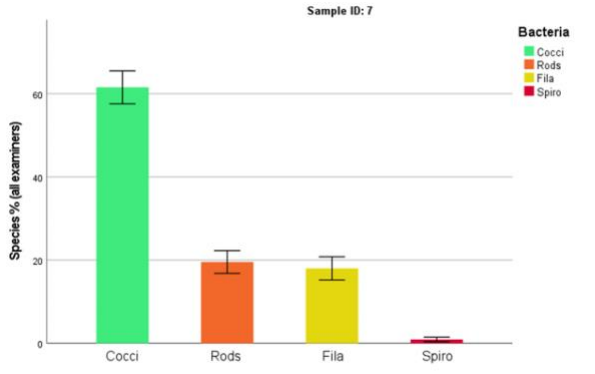


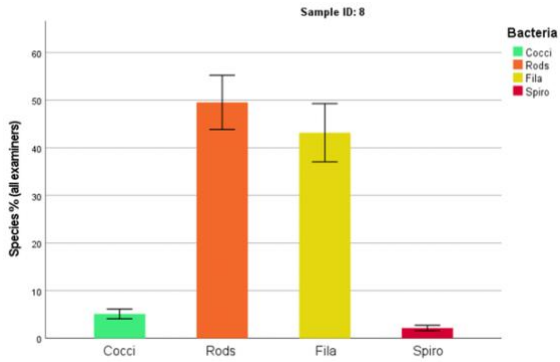


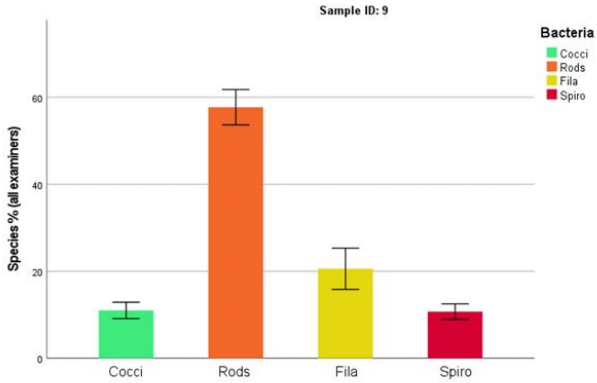


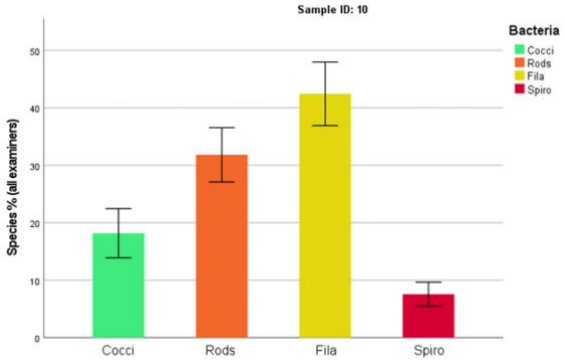


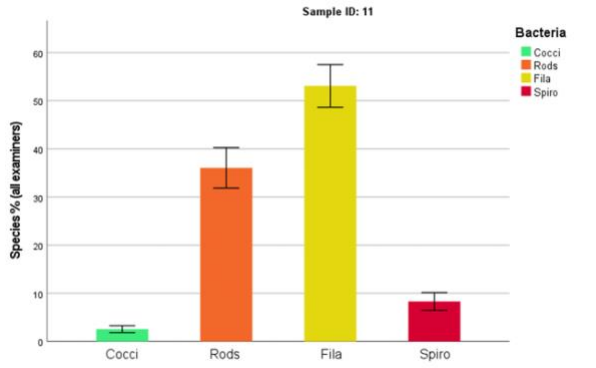


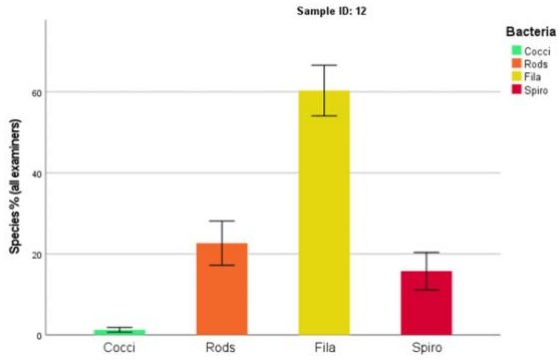

Sample ID: 7
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
20
40
60
Cocci
Rods
Fila
Spiro

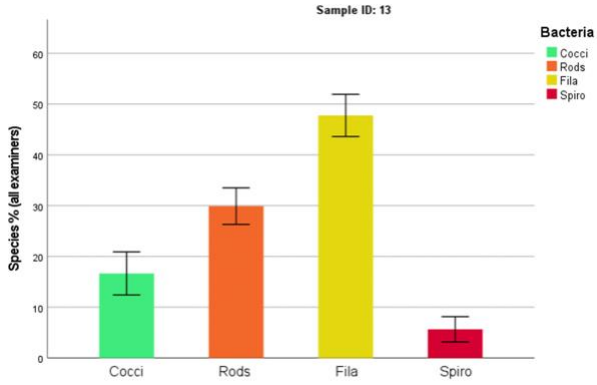

Sample ID: 8
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
10
20
30
40
50
60
Cocci
Rods
Fila
Spiro

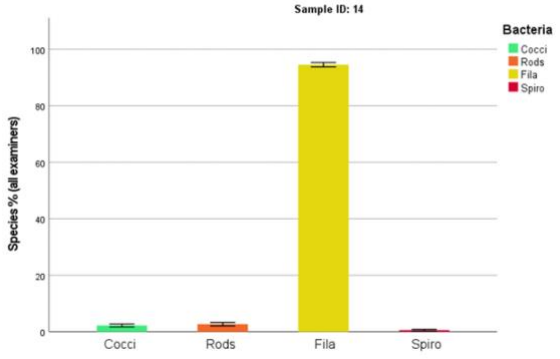

Sample ID: 9
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
20
40
60
Cocci
Rods
Fila
Spiro


Sample ID: 10
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
10
20
30
40
50
Cocci
Rods
Fila
Spiro


Sample ID: 11
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
10
20
30
40
50
60
Cocci
Rods
Fila
Spiro


Sample ID: 12
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
20
40
60
Cocci
Rods
Fila
Spiro


Sample ID: 13
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
10
20
30
40
50
60
Cocci
Rods
Fila
Spiro


Sample ID: 14
Bacteria
Cocci
Rods
Fila
Spiro
Species % (all examiners)
0
20
40
60
80
100
Cocci
Rods
Fila
Spiro