CHEMOTHERAPEUTIC DECONTAMINATION OF DENTAL IMPLANTS COLONIZED BY MULTISPECIES ORAL BIOFILM

by

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Abstract

Objectives: A recent meta-analysis reported that 18.8% of patients treated with dental implants are affected by peri-implantitis. Chemotherapeutic agents are often used during surgical decontamination of the dental implant despite limited evidence to support their efficacy. It is also known that mature biofilms are more resistant to antimicrobial agents. No studies have tested disinfectants on mature multispecies oral biofilms on titanium substrata. The aim of this study is to develop a multispecies oral biofilm implant model and to determine its susceptibility to antimicrobial agents. The null hypothesis is that no chemical agent is more effective than saline rinse to decontaminate sandblasted acid etched (SLA) titanium dental implant.

Methods: Collagen-coated SLA titanium discs were inoculated with dispersed dental plaque with minimum bacterial cell concentration of 3.2×10^7 CFU/ml. After 21 days of anaerobic incubation, discs were rinsed with 0.9% NaCl to remove unattached biofilm, and exposed for 2 minutes to tetracycline paste, 1% chlorhexidine (CHX) gel, 35% phosphoric acid gel. Discs were rinsed again to remove the chemical agents. Bacterial counts were quantified from standardized scanning electron micrographs of the implant surface. Disinfectants were compared within each other and with the control groups (rinse and double-rinse).

Results: After three weeks, the biofilm thickness on SLA discs was approximately 30 μm and showed the presence of multitude of rod and coccoid organisms. Rinsing the surfaces with 0.9% NaCl removed the majority of the biofilm. However, bacteria persisted in all specimens regardless of the treatment and none of the disinfectants was superior to the saline double-rinse group. CLSM analysis showed that CHX and Etch groups had a statistically significant reduction

of viable bacteria within the biofilms, although small. New chemical and peeling-off techniques were also tested but did not remove significantly more bacteria than the double-rinse group.

Conclusions: This mature multispecies biofilm model may be useful for the evaluation of decontamination of SLA implant surface. The tested chemical agents and the peeling-off techniques did not improve the decontamination effect when compared with the 0.9% NaCl rinse. CHX and Etch may provide a slight advantage in killing some of the remaining bacteria.

Preface

This dissertation is an original intellectual product of the author, Sébastien Dostie. The University of British Columbia's Research Ethics Board [certificate number H12-02430] approved all experimental methods. Sébastien Dostie, Dr. ZheJun Wang and Dr. Ya Shen collected the biofilm. Sébastien Dostie performed all experimental manipulation, and prepared the samples for SEM imaging. Dr. G. Owen carried out SEM imaging of all samples, while Dr. ZheJun Wang prepared the selected samples for CLSM and carried out imaging. Sébastien Dostie proceeded with the images analysis both for SEM and CLSM images, prepared the corresponding figures and all statistical analysis.

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List of Abbreviations

AL:	Attachment loss
APT:	Active periodontal therapy
BoP:	Bleeding on probing
BHI:	Brain heart infusion broth
CLSM:	Confocal Laser Scanning Microscopy
CHX:	Chlorhexidine
FMPE:	Full mouth periodontal examination
HA:	Hydroxyapatite
HMDS:	Hexamethydilazane
LPS:	Lipopolysaccharide
OR:	Odds ratio
PBS:	Phosphate buffered saline
PD:	Probing depth
PMPE:	Partial mouth periodontal examination
SEM:	Scanning Electron Microscopy
(SEM):	Standard error of the mean
SFE:	Surface free energy
SLA:	Sandblasted, Large-grit, Acid-Etched
SPT:	Supportive periodontal therapy

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Dedication

To My Children, Guillaume, Frédérique and Olivier:

Throughout my life, I have always strove to fulfill my potential in order to become the best person I could be and this doctrine is what motivated me to go back to university. With this decision however, it meant that the responsibilities necessary of a graduate resident resulted in spending less time with you. This was an incredibly painful sacrifice. This thesis is then dedicated to the three of you and may its completion help you understand and appreciate that each of you can realize whatever dream you cherish as long as you are ready to put forth the necessary hard work and discipline. I love you all, more than words can say.

Chapter 1: Introduction

Despite the high success rates of dental implants, osseointegrated implants are susceptible to peri-implantitis, which may eventually lead to dental implant loss.[1-4] Peri-implantitis is defined as mucosal inflammation and loss of supporting bone. It is infectious in nature and the oral microflora is the primary source of pathogens. Removal of bacterial biofilms is routine part of the treatment of peri-implantitis.[1] Due to the screw-shaped design and the microstructure of the implant surface, the mechanical debridement alone is incapable of removing all biofilms. Clinicians are often using different chemotherapeutic agents, such as chlorhexidine (CHX) and Tetracycline paste, as adjunctive treatment to mechanical debridement in order to decrease the microorganisms to a level compatible with periodontal health. Phosphoric acid gel has also been used to decontaminate dental implants during surgical treatment.[6, 7] However, there are limited in vitro studies supporting the use of one agent over the others.[8-12] Moreover, these studies did use single-species immature oral biofilms. It is known that distinctive subgingival microbiota become established 3 to 12 weeks after supragingival plaque formation,[13, 14] and that the maturity of the biofilms increase its resistance to anti-microbial agents.[15, 16] An in vitro biofilm model described by Shen et al[17] could potentially be used to assess implant surface decontamination. Using this model, it was also shown that the addition of a surface modifier to CHX killed bacteria much faster in anaerobic, multispecies biofilm than 2% CHX.[17]

To date, no studies have tested disinfectants on mature multispecies biofilms using titanium substrata. The aims of this study are to develop a biofilm model on Straumann® implant SLA surface that closely mimics in vivo biofilm and to determine its susceptibility to antimicrobial agents compared to saline rinse. A new chlorhexidine decontamination agent

formula will also be developed for a pilot study, as well as a new potential decontamination technique (peeling-off technique).

The null hypothesis is that the chemical agent or the peeling-off technique will not remove more mature multispecies bacterial biofilms from the dental implant surface compared to saline rinse only.

Chapter 2: Review of the Dental Literature

2.1 Prevalence and Severity of Periodontitis

Periodontitis is an infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment and bone loss.[18, 19] A recent study revealed that 47.2% of the American population over the age of 30 is affected by periodontitis.[20] The data was obtained from the National Health and Nutrition Examination Survey (NHANES) 2009-2010. In this study, severe periodontitis was defined as the presence of two or more interproximal sites with ≥ 6 mm AL (not on the same tooth) and one or more interproximal site(s) with ≥ 5 mm PD. Moderate periodontitis was defined as two or more interproximal sites with ≥ 4 mm clinical AL (not on the same tooth) or two or more interproximal sites with PD ≥ 5 mm (not on the same tooth). Mild periodontitis was defined as ≥ two interproximal sites with ≥ 3 mm AL and ≥ two interproximal sites with ≥4mm PD (not on the same tooth) or one site with ≥5mm. When taken separately, severe, moderate and mild periodontitis represented 8.5%, 30.0% and 8.7% respectively of the affected population. These estimations are considered more accurate than previous report since they used a full mouth periodontal examination (FMPE) protocol. Indeed, because of the uneven distribution of periodontal diseases in the mouth, a partial mouth periodontal examination (PMPE) underestimate disease in the population.[21] This is evident when comparing the estimated prevalence of periodontitis of 19.6% in the NHANES III (1988-1994) and the estimated prevalence of periodontitis of 27.3% in the NHANES 2000-2004, that both used PMPE. When the protocols used in these two studies are applied to the NHANES 2009-2010 data, prevalence of periodontitis is estimated to 19.5% and 27.1% respectively.

Unfortunately, the 2007-2009 Canadian Health Measures Survey (CHMS)[22] used a PMPE protocol and different criteria to defined the severity of periodontitis. Therefore, it is difficult to compare the prevalence of periodontitis in the Canadian population with the latest reported prevalence in the US population. The Canadian study used the World Health Organization's indicator teeth (1st and 2nd molars of all quadrants, an upper and lower anterior tooth) and measured PD and AL at six sites per tooth. Periodontal disease in this survey was assessed by the extent of AL: 79.1% of dentate adults were considered to have good periodontal health (0 to 3mm of AL), 15.1% have or have had moderate periodontal disease (4-5mm of AL) and 6.0% have or have had severe disease (6mm or more of AL).

Despite the difference in the disease definitions and the assessment protocol, the estimated prevalence of advanced periodontitis for both the latest American and Canadian survey are similar, 6.0% and 8.5% respectively. Although AL is used to assess the periodontitis, the true endpoint of the disease is tooth loss.

2.2 Periodontitis and Tooth Loss

From the 1981 U.S. household survey of Dental Health Related and Process Outcomes Associated with Prepaid Dental Care, it was estimated that periodontal disease in the U.S. adult population aged 19 years and older was the cause of less than 20% of all missing teeth and that extraction was required in 4% of all persons due to advanced periodontal destruction.[23] However, it is also interesting to note that the effect of periodontal disease on tooth extraction increase with age and reached 28.7% for the 65 and more years old group, when compared to 6.9% for age 19-44 and 16.8% for 45-64 years old group.

The reasons for tooth extractions in general dental practice in Ontario was assessed via record forms for each patient seen during a reference working week.[24] Out of 461 Ontario general practitioners, 128 returned 6134 completed patient record forms. From these patients, 867 had an extraction or were scheduled to have an extraction as part of the current treatment plan. Caries accounted for 28.9% of permanent teeth extraction, while periodontal disease was the reason for 35.9% of the extraction. Between 20 to 39 years of age, caries was the main reasons for extraction at 33.3% and remained stable over the years. Periodontal disease accounted for 19.3% in the young adult population but reach 60.6% for the 40-59 years old group and 46.5% for 60 years and older. The authors claimed that extractions due to caries are less likely to occur in regular patients like it was the case for this study (88.4% of the patients were regular). This is especially true when only 7% of services delivered to Ontarians aged 50 years and older were for periodontal treatment, compared to over 40% for restorative purposes.[25]

A similar study was conducted in Japan ten years later.[26] A questionnaire was sent to 5131 general dental practitioners requesting to collect data for one working week on the reasons of permanent tooth extractions. Data was obtained from 2001 dentists: 9115 teeth were extracted from 7499 patients. The largest number of teeth extracted was in the 55-64 years of age. Overall, 43.4% were extracted due to caries, 41.8% were extracted due to periodontal disease, 1.2% for orthodontic reasons and 13.6% for 3rd molars extraction and prosthetic reasons. Caries or fractures were the main reasons for extractions in the group aged from 25-34 and 35-44 with 50.1% and 57.3% respectively. However, periodontal disease was the main reason for extraction in the group age of 45-54 and older (approximately 50%).

The above-mentioned studies did not consider periodontal treatment into their analysis of tooth loss. In a retrospective cohort study from Matuliene et al,[27] the odds ratio (OR) of loosing tooth at site and tooth-level were ranging from 2.6 and 2.5 after active periodontal therapy (APT) for PD of 4mm, to 37.9 and 64.2 for PD of 7mm or more. Smoking also had an OR ranging from 1.8 (1-19 cigarettes/day) to 5.9 (20 or more cigarettes/day) of periodontal disease progression.

Ravald and Starkhammar Johansson[28] studied the periodontal condition, tooth loss and reason of tooth loss of patients who has been treated and maintained for two years in periodontal specialty clinic before being referred back to the general dental practitioner to pursue supportive periodontal therapy (SPT) program. Sixty-four patients were available for the re-assessment 11 to 14 years after APT and SPT performed at the specialty clinic. A total of 211 teeth were lost over the observational period, mean of 3.3 teeth per patient. However, 24 patients did not loose any tooth, therefore, 40 patients lost a mean of 5.3 teeth per patient. Moreover, 17 patients accounted for 167 lost teeth (77%), or 9.8 teeth per patient. The main reason for tooth loss was periodontal disease (153 teeth). An OR of 1.11 was found between PD of 4-6 mm and tooth lost, while it was 8 for smoking. At the re-examination, 31% were classified as healthy, 45% showed periodontal problem, and 20% showed a combination of caries and periodontal diseases. The mean plaque score was 39% at the re-examination while it was 23% at baseline. It was argued by the authors that daily plaque control and the quality of the SPT were not sufficient, which made them concluded that for periodontally treated patients, the maintenance phase performed in general practice seems not to be sufficiently effective for prevention of tooth loss. However, there is no data other than the OR with regard to smoking. Since 17 patients accounted for 77%

of the tooth loss observed in this study, question can be raised whether if those 17 patients were smokers? This is particularly important when comparing the OR of 1.11 for PPD 4-6mm.

In a recent prospective cohort study, Costa et al[29] followed 265 individuals with chronic moderate to severe periodontitis who had undergone APT over a 5-year period. They showed that irregular complier patients had 3 times more chance of loosing teeth when compared to regular compliers. Moreover, the most common reason for tooth lost was periodontal disease (≈80%).

In light of the studies presented so far, periodontitis is highly prevalent worldwide with advanced periodontitis affecting 6 to 8% of the American and Canadian population. It is estimated that periodontitis is the main reason of tooth loss in about 20 to 40% of the individuals depending on the population studied. The studies also present a similar pattern of increasing percentage of tooth loss due to periodontitis with increasing age. Although not limited to the following factors, tooth loss in patients treated for periodontal disease is affected by their compliance to the treatment (SPT) and their smoking habits.

The 2007-2009 Canadian Health Measures Survey 2007–2009[22] do not provide any information about the reasons of tooth loss, However, it was found that 6.4% of adults (20-79 years old) are edentulous with minimal gender variation; this percentage increase to 21.7% for the adults aged between 60 to 79 years old. The percentage of adults that have all their teeth (excluding the 3rd permanent molar) is 43.3%, while 14.6% have fewer than 21 teeth. Only few edentulous (4.1%, 60-79 years old) and dentate (0.8%) patients had at least one implant. Although the high variability and the low number of patients with at least one dental implant

preclude any definitive conclusion from this survey, implants are more and more used to replace missing teeth and to support dental prosthesis.

2.3 Dental Implant Outcomes

2.3.1 Success and Survival Rates

Dental implants are widely used and provide good treatment option in a variety of clinical situation. Their success and survival rates have been reported in many investigations. The classic study from Albrektsson et al[30] showed that cylinder Branemark implants had a high success rate ranging from 99% in the mandible to 93% in the maxilla after 5 years. The success criteria used at that time were as following: absence of implant mobility when tested clinically; no evidence of peri-implant radiolucency on radiograph; vertical bone loss less than 0.2mm annually following the implant's first year of service; and absence of signs and symptoms such as pain, infections, neuropathies, paresthesia or violation of the mandibular canal.[31]

Using the same implant system and success criteria, Lekholm et al[32] found cumulative survival rates of 93.7% and 90.2% and for the mandible and maxilla respectively 10 years after completion of the prosthetic treatment in 127 partially edentulous patients. However, the individual implant stability was not tested since no prosthesis was removed at the 10-year follow-up, explaining why the authors used the term survival rates instead of success as described by Albrektsson.[30, 31]

Simonis et al[33] evaluated the long-term results of Straumann titanium plasma-sprayed (rough, microporous surface) dental implants in partially edentulous patients. They used the success criteria by Smith & Zarb,[34] which are the Albrektsson's criteria[31] with the addition of the following criteria: the implant allow the placement of a crown or prosthesis which is

aesthetically satisfactory to the patient and dentist; and a minimum success rate of 85% at the end of a 5-year observation period and 80% at the end of a 10-year period. From the 55 patients with 131 implants who were re-assessed 10 to 16 years after implant placement, 22 implants were lost; the survival rate was 89.23% after 10 years and the cumulative survival rates was 82.94% after 16 years. They also found that the prevalence of biological complications was 16.94% while it was 31.09% for technical complications.

More recently, Chappuis et al[35] also investigated the long-term results of the Straumann titanium plasma-sprayed dental implants in partially edentulous patients. The outcomes achieved were evaluated with both Albrektsson's success criteria and the Buser's success criteria. These later criteria are: no subjective complaints, no peri-implant infection with suppuration, no mobility, no peri-implant radiolucency and a possibility for restoration.[36] From the original cohort of 98 patients, 67 were available for re-examination after 20 years of function, in which 95 implants were placed in the 80's. A total of 10 implants failed and 19 implants had biologic complication from which 13 were successfully treated but were accounted as surviving implant (13.7%) but not successful implant. Following the Albrektsson's criteria, success was achieved in 89.5% of the implants, but only in 75.8% according to Buser's criteria. The bone level was stable in 92% of the surviving implants (78/85), which showed peri-implant bone loss lesser than 1mm over the 20-year observation period. This is interesting since the titanium plasma-sprayed (TPS) implants have been associated with significantly more bone loss after two to three years when compared to machined implants from Branemark,[37] while no difference in bone loss was observed between moderate roughness implant's surface and the machined surface.[38]

Dental implant surface have been modified (increased roughness) over the years to facilitate bone ingrowth into minor surface irregularities and therefore to improve bone-to-implant contact and osseointegration.[39] Roughness is usually expressed as surface area roughness (Sa). Profile roughness (Ra) can be extracted as a line through an area and is therefore a two-dimensional counterpart of the three-dimensional descriptor Sa. The Sa values varied between 1.0 and 2.0 μm for the TiOblast, SLA and TiUnite implants, while it varies between 0.5 and 1.0μm for the machined or turned type of implants. According to a systematic review from Albreksson and Wennerbeg,[40] moderately rough surface implants (Sa between 1.0 and 2.0μm) have shown stronger bone responses than smoother or rougher surfaces, improving the survival rates even more then previously reported.

In a retrospective study from Buser et al (2012),[41] survival rate of 98.8% and success rate of 97% were found after 10 years for 511 SLA implants placed in 303 patients. A recent retrospective study, also reported very high cumulative survival rates of 99.3%, 99.0%, and 98.4% at 3, 5, and 7 years when were respectively.[42] In this study, an impressive total of 4591 SLA or SLActive implants were placed in 2060 patients by a single periodontist in private clinic settings.

Generally speaking, high cumulative survival rates at 10 years or more ranging from 95% to 97% are reported for three of the major implants systems (TiUnite, TioBlast and SLA implants) using moderately rough surface.[43] However, there is still no available study with a follow-up of 15 to 20 years. Moreover, it is extremely difficult to compare the survival and success rates since there is no consistency and homogeneity of the parameters used to described them. Also, the success rate decrease as the number of parameters used to assess the success rate

increase.[44] Nonetheless, in light of the literature, implants provide good treatment options for completely or partially edentulous patients.

2.3.2 With Past History of Periodontitis

It has been hypothesized that an increased susceptibility for periodontitis might translate to an increased susceptibility for peri-implantitis and implant loss. In a prospective longitudinal study in which peri-implantitis was defined as an incidence of PD equal to or deeper than 5 mm with BoP and radiographic signs of bone loss, a failure rate of 9.5% was seen in the group with periodontitis history while it was 3.5% in the control group without history of periodontitis.[45] Even though this difference was not statistically significant, the group with past history of periodontitis had significantly higher incidence of peri-implantitis when compared to the control group. Indeed, peri-implantitis occurred in 28.6% of these individuals while it occurred in 5.8% in the control group. Furthermore, smokers with history of periodontitis were more affected by biological complications.

Fardal and Linden[46] investigated different factors associated with continuous tooth loss caused by periodontal diseases during maintenance therapy. From a group of 1251 patients that underwent active periodontal therapy and maintenance therapy for at least 8 years in a specialty clinic, 27 patients lost at least 10 teeth during the maintenance phase and were classified as refractory to periodontal therapy. Not surprisingly, they found that heavy smoking (20 cigarettes / day or more) and family history of periodontitis were strongly associated with tooth loss. Implants were placed in 14 patients of the refractory group. Although the study did not aim to assess implant survival in refractory patients, 64% of the refractory patients lost at least one implant, and 25% of all implants were lost in this group.[46] Interestingly, 21% of the implants

were never osseointegrated and were removed, while 3 implants (4%) were lost following peri-implantitis after osseointegration. Only 2 implants were placed in the control group, but both were successful.

A long-term cohort study from Levin et al[47] evaluated the implants' survival rates in relation to the periodontal status and other factors such as smoking and diabetes. The cohort consisted of 736 patients who received a total of 2336 implants from 1996 to 2006. They found the effects of smoking and periodontal status were not constant throughout the follow-up period. Up to 50 months, no difference in the survival rates was noted between periodontal status and smoking status. After 50 months, severe chronic periodontitis and smoking were found to be strong risk factors with hazard ratios of 8.06 ($p<0.01$) and 2.76 ($p=0.061$) respectively while controlling for the smoking or periodontal status effects, separately. However, patients with moderate chronic periodontitis were not at a higher risk of implant failure when compared to periodontal healthy patients. The authors stipulated that the greater risk of implant failure in patients with severe chronic periodontics after 50 months was due to continuous and cumulative nature of periodontal disease. Nonetheless, this study clearly showed that a minimum of 5 years follow-up is necessary to assess the real effect of periodontal status on implant survival.

Similarly, Roccuzzo et al[48] found that periodontally compromised patients presented a lower implant survival rate than periodontally healthy patients after 10 years but this difference was not statistically significant. Although there was no statistical difference between the groups with regard to the mean bone loss, severe periodontally compromised patients had significantly more sites with bone loss of 3mm or more. Furthermore, moderate and severe periodontally compromised patients had significantly more implant loss if they were not fully adherent to their

personalized SPT program.[48] In a following paper,[49] the same group of authors found that periodontally compromised patients had more biological complication, which required more cumulative interceptive supportive therapy[50] when compared to periodontally healthy patients.

In light of the above-mentioned long-term studies, it can be suggested that periodontally compromised patients are more prone to biological complications, peri-implant diseases, and are at higher risk of implant failure.

2.4 Epidemiology and Risk Factors of Peri-implant Diseases

Peri-implant diseases can be defined as inflammatory lesions around dental implant. This term includes peri-implant mucositis and peri-implantitis. The first one is described as the presence of inflammation in the peri-implant mucosa with no signs of loss of supporting bone. On the other hand, peri-implantitis is the presence of inflammation of the mucosa and loss of supporting bone.[5]

The usual periodontal clinical parameters are used combined with radiographs to diagnose peri-implant diseases. Animal studies showed that increased of probing depth over time was associated with attachment and bone loss.[51, 52] Bleeding on probing (BoP) around dental implant indicates the presence of inflammation[53] but can also be a sign of disease progression at site when BoP is present at more than half of the recall visits over a 2-year period.[54] Suppuration was associated with bone loss around implant resulting in exposure of 3 or more threads in a report of 218 patients.[55] Implant mobility is not useful for early diagnosis of peri-implant diseases since it is a sign of complete lack of osseointegration.

After 9 to 14 years of function, Roos-Jansaker et al[56] found prevalence of 76.6% and 16% for peri-implant mucositis and peri-implantitis at the patient level. The prevalence at the implant level was 48% and 6.6%, respectively. The same group of authors found that smoking was associated with mucositis and peri-implantitis, and peri-implantitis was related to a previous history of periodontitis.[55]

Ferreira et al[57] investigated the prevalence of peri-implant diseases in partially edentulous patients. From the 212 patients included in the study, 64.6% and 8.9% were affected by peri-implant mucositis and peri-implantitis, respectively. They also found that periodontitis and diabetes were statistically associated with an increased risk of peri-implantitis with respective OR of 3.1 and 1.9; however, no association was found between maintenance's frequency and the prevalence of peri-implant diseases. On the other hand, Rinke et al[58] found a statistical significant association between peri-implantitis and smoker (OR: 31.58), but also with non-compliant patients to SPT (OR: 11).

The studies from Rinke et al[58] and Mir-Mari et al[3] found similar prevalence of peri-implant diseases when investigating data from two private practices. Peri-implant mucositis was diagnosed in 38.8% and 44.9% of the patients, while peri-implantitis was diagnosed in 11.2% and 16.3% of the subjects. However, peri-implantitis has been estimated up to 37% in a recent cross-sectional study conducted in a Belgian population.[2]

These variations of the prevalence of peri-implant diseases reported in the literature, both at the subject and implant level, are mainly depending on the diagnostic thresholds used to define the diseases. Koldsland et al[59] clearly showed that the prevalence of peri-implantitis could vary

from 11.3% to 47.1% within the same population, just by modifying the probing depth and the radiographic peri-implant bone loss used to diagnose the peri-implantitis.

Other factors may also be responsible for such reported variations of peri-implantitis prevalence. As such, the implant types should be considered when reporting the prevalence of peri-implantitis according to Albrektsson et al.[43] In their critical review of the literature, these authors conclude that modern implants, such as SLA, TiUnite, and TiOblast, provide a higher success rates then turned implants, but also a lower prevalence of peri-implantitis, as low as 1 to 2% of placed implants. They also conclude that the higher rates of peri-implantitis prevalence reported in the literature is over-estimated and mainly due to the three "poor": poorly trained surgeon, poor quality of implant, and poor patient selection. Because not all "poor" can be excluded, it is likely that peri-implantitis has much higher prevalence than 1 to 2 %.

Actually, a recent meta-analysis reported that 63.4% and 18.8% of patients treated with dental implants are affected by peri-implant mucositis and peri-implantitis respectively.[60] At the implant level, the same study found that the frequency of peri-implant mucositis is estimated to be 30.7%, whereas peri-implantitis is estimated at 9.6%. Just like periodontal diseases, a higher frequency of occurrence of peri-implant diseases was recorded for smokers, and supportive periodontal therapy seems to reduce the rates of occurrence. It is generally accepted in the literature that poor oral hygiene, history of periodontitis and cigarette smoking are associated with peri-implant diseases, poor metabolic control (diabetes) could also be a risk factor of peri-implantitis.[61]

2.5 Anatomy: Similarities and Differences between Natural Tooth and Dental Implant

Despite general similarities, many differences exist between the anatomy of peri-implant structures and a natural tooth, which are helpful to understand the difference of progression between periodontitis and peri-implantitis.

The soft tissue surrounding a natural tooth is called gingiva, while it is called peri-implant mucosa around a dental implant. Healthy peri-implant mucosa is crucial to achieve long-term implant survival, but also high aesthetic result. Using submerged dental implant in the beagle dog model, Berglundh et al[62] showed that peri-implant mucosa established a cuff-like barrier that adhered to the surface of the titanium abutment, similarly to the gingiva around natural tooth, and seal the underlying bone from the oral cavity. The peri-implant mucosa, or the gingiva around natural tooth, consists in an oral epithelium, a sulcular epithelium, connective tissue, and a junctional epithelium.

The sum of the connective tissue and the junctional epithelium around natural teeth form the biological width. This term was based on the work of Gargiulo et al[63] who described the dimensions and relationship of the dentogingival junction in humans. Measurements made from the dentogingival components of 287 individual teeth from 30 autopsy specimens established that there is a definite proportional relationship between the alveolar crest, the connective tissue attachment, the epithelial attachment, and the sulcus depth. They reported the following mean dimensions: a sulcus depth of 0.69 mm, an epithelial attachment of 0.97 mm, and a connective tissue attachment of 1.07mm. Based on this work, the biologic width is commonly stated to be 2.04 mm. Similar biologic width dimensions were also reported by Vacek et al[64] who evaluated 171 cadaver tooth surfaces, they observed mean measurements of 1.34 mm for sulcus depth, 1.14

for epithelial attachment, and 0.77 mm for connective tissue attachment. Both groups found significant variations but observed that the connective tissue attachment was the most consistent measurement.

The biological width around one-piece dental implant has been reported to be similar to the dimensions of the dentogingival tissues and stable over time.[65, 66] The peri-implant epithelium around non-submerged one-piece dental implant was 1.88mm while the connective tissue was 1.05mm.[66]

Around two-pieces dental implants (butt-joint interface), the biological width has been reported to be approximately 2.14mm from the gingival margin to the most apical portion of the peri-implant epithelial attachment (2.05mm on control teeth) and 1.66mm connective tissue attachment around the implant (1.12mm on control teeth).[62] The connective tissue was significantly thicker around the dental implant when compared to natural tooth. This resulted in a mean supracrestal soft tissue height of 3.17 mm at teeth and 3.80 mm at dental implants, which was also significantly different.

Interestingly, crestal bone resorption will occur when the peri-implant connective tissue has been previously excised or when the implant is surrounded by a thin peri-implant mucosa. This phenomenon is believed to allow for attachment of appropriate tissue thickness, or biological width.[67, 68]

Similarly a butt-joint interface will promote more bone resorption when compared to a platform-switching interface, resulting in an increased biological width. The platform switching moves the implant-abutment interface horizontally towards the center of the long axis of the

implant. This type of connection provide an increased surface area for more attachment of the peri-implant soft tissues, and moves the inflammatory cell infiltrate that is associated with the implant abutment interface away from the bone crest.[69] More recently, an animal study of the biologic width dimensions around implants with platform switching demonstrated a healthy, inflammation-free connective tissue covering the implant abutment interface, while it has always been reported to be more apical to the interface in a butt-joint interface.[70] The authors hypothesized that the platform-switching interface minimizes bacterial colonization, therefore reducing or eliminating the bone destructive effect of a bacterially induced inflammation.

While oral and sulcular epithelium are only connected to the underlying connective tissue, the junctional epithelium is unique because it faces the underlying connective tissue of the gingiva but also the tooth surface. The junctional epithelium is part of the free-gingiva and is a stratified squamous non-keratinizing epithelium. It consists of only two zones: a single cell layer of cuboidal cells (stratum basale) and several layers of more flattened cells equivalent to stratum spinosum. The suprabasal cells of the junctional epithelium facing the tooth surface are called DAT cells (directly attached to the tooth) and they form the internal basal lamina. The epithelial attachment between the tooth surface and the junctional epithelium is achieved by the internal basal lamina together with the hemidesmosomes.[71, 72]

The junctional epithelium around a dental implant is referred to peri-implant epithelium and always originates from the oral epithelium (epithelial downgrowth) during the healing process, while the junctional epithelium around natural teeth originates from the fusion of the reduced enamel epithelium with the oral epithelium.

The peri-implant epithelium was first reported to be very similar to the junctional epithelium and its presence around dental implant was confirmed in many studies.[66, 73] For both, the width of the junctional epithelium around implants and natural teeth is greatest at its coronal aspect and decreases apically. Similar to epithelial attachment around natural tooth, Gould et al[74, 75] observed under electron microscope the peri-implant epithelium and found that it was attached to titanium implant via hemidesmosomes, both in vitro and in vivo (human). However, Ikeda et al[76] observed in a rat model with transmission electron microscopy and immunoelectron microscopy the peri-implant epithelium, and found hemidesmosomes only in the most apical portion of the interface, which adhere less than around natural tooth. Using the Beagle dog model, another study showed the presence of bacteria between the dental implant and the peri-implant epithelium but failed to observe the presence of hemidesmosomes, once again supporting previous observation of a weaker epithelial attachment to the titanium implant surface when compared to junctional epithelium around natural teeth.[77] Another study failed to show the presence of hemidesmosomes and basal lamina at the interface between the epithelium and the implant eight weeks after implantation in rats, and the peri-implant epithelium showed characteristics of the oral sulcular epithelium.[78] Using the rats model, more recent studies also suggest a weaker connection of the peri-implant epithelium when compared to the junctional epithelium, and this junction is even weaker with rough-surfaced implant compared to machine-surfaced implant.[79, 80] Therefore, it is suggested to use smoother surface on the supra-bony portion of the implant or on any surface that may become exposed after bone remodelling.[79] In light of these recent animal studies, it seems like the peri-implant epithelium differ from the junctional epithelium around natural teeth, and may be weaker to oral insult.

The zone between the apical portion of the peri-implant epithelium, or the junctional epithelium, and the coronal portion of alveolar bone is filled by connective tissue. Unlike natural tooth, dental implant lacks root cementum and functionally oriented Sharpey's fibres that insert into it. The collagen fibres in the connective tissue around natural tooth originate from the cementum and reach the alveolar bone and the gingival tissue in a fan-shaped fashion. On the other hand, the collagen fibres originating from the marginal bone are parallel with the abutment surface of submerged implant, without evidence of fibres insertion at the implant or abutment interface.[62] Similar connective tissue orientation was found around non-submerged dental implant and abutment.[81, 82] In addition to the parallel orientation of the connective tissue, other studies found collagen fibres that are oriented in a circular pattern around the dental implant in both humans[83] and animals.[62, 73]

Although soft tissue attachment on dental implant or abutment was shown not to be influenced by the titanium roughness surface,[82] a canine study showed that laser ablated microgrooves in the abutment or the collar of a dental implant resulted in perpendicularly oriented connective tissue fibres similar to that around natural teeth (Sharpey's fibres), i.e. at 90^{o} insertion to the abutment or implant surface.[84] This is thought to create a soft tissue seal around dental implant, which inhibit the apical migration of the epithelial cells and helps to minimize crestal bone remodelling.[85]

Many of the above mentioned studies have described two distinct supracrestal zones of connective tissue around dental implants.[73, 81, 82] The inner zone of the connective tissue, adjacent to dental implant, was described as poorly vascularized and resembled a scar-like tissue when compared to the more peripheral connective tissue. The inner zone was later described as rich in

fibroblasts and it was hypothesized that this fibroblast rich zone was playing a crucial role in the maintenance of proper seal between the peri-implant bone from the oral cavity.[86] The peripheral connective tissue zone was described on the other hand as a well organized but less dense collagen fibres network with a lower proportion of fibroblasts but that was richly vascularized. However, the connective tissue in a 100 μm wide zone surrounding a natural tooth had a lower proportion of collagen fibres (76.4% vs. 87.0%) and vascular vessels (2.6% vs. 5%) with a higher proportion of fibroblasts (5.4% vs. 3%) when compared with connective tissue surrounding dental implant, and these difference were statistically significant.[62] Following the hypothesis presented by Moon et al,[86] a higher proportion of fibroblasts around natural teeth may suggest better connective tissue seal.

In summary, despite some resemblances (e.g., the re-establishment of a biological width around implant and cuff-like barrier seal), the peri-implant mucosa (connective tissue and peri-implant epithelium) do not attached as firmly around dental implant when compared to natural tooth.

2.6 Pathogenesis and Progression of Peri-implant Diseases

2.6.1 Peri-implant Mucositis

As previously discussed, the term peri-implant mucositis is described as the presence of inflammation in the mucosa at an implant with no signs of supporting bone loss.[5] This term was defined to comply with the existent gingivitis definition due to its similarities with this periodontal disease. Therefore, it was not surprising that researchers used periodontal disease existing study model to study peri-implant diseases.

The gingivitis model developed by Löe et al[87] showed the cause and effect relationship between plaque accumulation and gingivitis. Using this model, the same relationship between dental plaque accumulation and peri-implant mucositis was found.[88] In this study, 20 partially edentulous patients received full periodontal treatment and one dental implant restoration in the posterior areas. After baseline examination, the patients were asked to refrain from oral hygiene practices in the area of the implant and adjacent teeth for a period of three weeks. Gingival index (GI), plaque index (PlI), sulcus bleeding index, probing depth (PD) and recession were measured. Submucosal and subgingival plaque samples were also obtained. After three weeks of plaque accumulation, all the clinical parameters around the implant and adjacent control tooth showed a statistically significant increase when compared to the baseline results. However, there was no significant difference between the implant and the tooth. Similarly, the phase-contrast microscopic analysis did not show significant differences in the compositions of submucosal biofilms around implants when compared subgingival biofilms at tooth sites. However, a higher proportions of spirochetes and motile rods and lower proportions of coccoid bacteria were found after three weeks of refraining from oral hygiene, both at implant and tooth sites.

Another study in 12 partially dentate subjects confirmed the cause-effect relationship between the dental plaque and peri-implant mucositis.[89] Biopsies were taken at baseline and after 3 weeks of plaque accumulation. The results indicate that plaque accumulation induced an inflammatory response characterized by increased proportions of T-cells and B-cells in the infiltrated connective tissue of both the gingiva and the peri-implant mucosa. In sites with peri-implant mucositis, the inflammatory cell lesion was dominated by T-cells and had an apical

extension that was restricted to the barrier epithelium. However, no significant differences in the composition of the host response were noted between these two tissues.

These last two studies provided the first accepted proof of cause-effect relationship between dental plaque accumulations and peri-implant mucositis. However, this relationship cannot be completely established without confirming the reversibility of the inflammation lesions. Also using the human gingivitis model, Salvi et al[90] showed that the gingival index was significantly higher around implants when compared to the control teeth at all time during the experiment. This difference was also associated with higher levels of matrix-metalloproteinase-8 (MMP-8) around implants, a marker of neutrophil function indicating an active phase of inflammation. After three weeks of plaque accumulation, the 15 subjects were asked to resume oral hygiene. Despite the fact that biomarkers came back to baseline level after three weeks of plaque control, the gingival index was still higher, both around implants and teeth. The authors concluded that the reversibility of the dental plaque induced gingivitis and peri-implant mucositis required more than 21 days to reach healthy status, and that the inflammatory response to dental plaque accumulation was stronger around dental implant. Despite this stronger reaction of peri-implant mucosa to dental plaque, it is well accepted that the description of the inflammatory process of peri-implant mucositis around an implant is quite similar to gingivitis around natural teeth.[91]

2.6.2 Peri-implantitis

Peri-implantitis is the destructive inflammatory processes around osseointegrated implants that lead to peri-implant loss of supporting bone. It is infectious in nature and the oral microflora is the primary source of pathogens.[5, 92, 93]

Contrary to peri-implant mucositis, the peri-implantitis lesion extended apical to the pocket epithelium. Furthermore, B cells outnumbered T cells in the peri-implantitis lesions and the proportion of B cells was three times larger in peri-implantitis than in mucositis sites. PMN cells and macrophages were also found in high numbers in different areas of peri-implantitis lesions.[92]

Pathological differences were also noted between peri-implantitis lesions and periodontitis lesions. Both lesions are characterized by a large inflammatory cells infiltrate adjacent to the pocket epithelium, but peri-implantitis lesions contain a higher proportion of neutrophils and macrophages and have a more pronounced apical extension that goes beyond the pocket epithelium.[94, 95]

A study using ligature allowing dental plaque accumulation in dog showed that tissue destruction were more pronounced, and that the size of the inflammatory cell infiltrate in the connective tissue was larger around implants than teeth.[93] A study in monkey found similar findings, but also that the bone loss was associated with the histological finding of numerous osteoclasts.[52] From these studies, it was also noted that the peri-implantitis lesions progressed more quickly than the periodontitis lesions. The rapidity of the peri-implantitis progression could be explained by the lack of inserting collagen fibres into the implants.[82, 96]

It was also noted in these animal studies that the ‘‘self-limiting’’ process that occurred in the periodontal tissues after ligature removal did not take place in the peri-implant tissues, but rather a continuous progression of the disease and subsequent bone resorption was observed. In another study using ligature breakdown model in dog, large inflammatory cells infiltrate extending to the bone crest was still observed one month after ligature removal.[97] Furthermore,

grooves and cavities with osteoclasts were frequently found on the bone crest suggesting bone active resorption process. Three months after ligature removal, a dense connective tissue capsule separated the peri-implantitis lesions from the bone crest in two of the three dogs sacrifice at 3 months. However, three of the four implants were lost due to continuous breakdown after ligature removal in the third dog and the fourth implant in this animal showed extensive bone loss and the peri-implantitis lesion extending into the marrow spaces of the peri-implant bone. Osteoclasts were still found on the surface of the bone crest three months after ligature removal. This lack "self-limiting" process is believed to be associated with the fact that inflammatory cells were frequently found in the infiltrated connective tissue area and were in direct contact with the biofilm or the layer of pus,[98] whereas the biofilm and the inflammatory cells are usually separated by the pocket epithelium in periodontitis lesions.

Using the monkey ligature breakdown model, it was also determined that probing measurements around osseointegrated dental implants and teeth were different. Indeed, it was shown that the probe tip was closer to bone around implants than around teeth in conditions of mild mucositis versus gingivitis, severe mucositis versus gingivitis and peri-implantitis versus periodontitis.[96] These differences were all statistically significant. More importantly, the histological examination demonstrated similar health and inflammation status around implants and teeth. Therefore, the difference in probe to bone distances between implants and teeth can be related to their different marginal connective tissue configuration; the absence of cervical cementum with inserting connective tissue fibres around dental implant may facilitate probe penetration.

As it was pointed out so far, the role of biofilms in peri-implant diseases is essential for the development of infections around dental implants. However, it is important to consider the possibility that peri-implant diseases may also arise as a consequence of non-microbial events favouring the emergence of a pathogenic microbiota. The most common example is the peri-implant infection due to submucosal excess of cement. Using a dental endoscope, excess of dental cement was associated with clinical and/or radiographic signs of peri-implant disease in 34 of the 42 test implants (81%), and the clinical signs of disease were gone in 74% of the test implants one month after the excess cement was removed.[99] Another retrospective study found similar prevalence of peri-implantitis (85%) when cement remnant was present.[100] In another study analyzing 36 biopsies of peri-implantitis under light microscope, foreign material resembling dental cement was noted in 8 biopsies, and foreign material that did not appear to be dental cement was seen in 9 biopsies.[101] Further analyses were conducted with scanning electron microscope equipped with energy dispersive X-ray spectrometry, and they found that 19 biopsies of 36 cases contained particles that are characteristically present in dental cements. Interestingly, a recent systemic review and meta-analysis did not find differences in marginal bone loss between cement and screw-retained implant-supported restorations.[102] However, the presence of residual cement was not assessed in this study.

Another factor that was reported to impact the progression peri-implant diseases is occlusal overload. For ethical reason, the effect of overloading forced on implants and bone resorption have been mainly study in animal models. In the presence of peri-implant mucosal health, excessive occlusal load did not result in loss of osseointegration or marginal bone loss but also significantly increased the bone implant contact.[103, 104] However, overloading implants with

ligature-induced peri-implantitis significantly increased the exposure of implant thread exposure due to buccal and lingual vertical bone resorption in the dog model.[104] These findings were both corroborated and contradicted in the monkey model.[105, 106] Nevertheless, it is usually accepted that overload does not cause peri-implantitis, that supra-occlusal contacts acting in an healthy peri-implant environment do not negatively affect osseointegration and are even anabolic, while supra-occlusal contacts in the presence of inflammation significantly increased the plaque-induced bone resorption.[107]

2.7 Biofilm

2.7.1 Around Tooth

Dental biofilms are complex, multispecies ecosystems, where oral bacteria interact cooperatively or competitively with other members. Approximately 75% of the volume of dental plaque is composed of bacterial cells, while 25% consists of epithelial cells, leukocytes, and macrophages in an intermicrobial matrix.[108]

The growth and maturation patterns of bacterial plaque have been studied on natural tooth surfaces, such as enamel and dentin, and also artificial surfaces such as epoxy resin crowns. Listgarten[13, 14] and Zijnge et al[109] observations on biofilms formation are briefly summarized in the following paragraphs. After a tooth surface is cleaned, the formation of supragingival plaque is initiated within minutes by formation of an acquired pellicle composed of salivary glycoproteins. This will promotes adhesion of Gram-positive facultative cocci and cocco-bacilli (*Streptococcus, Actinomyces, Veillonella*) as the initial colonizers. Dividing bacteria will spread laterally and then grow in thickness, forming closely packed microbial colonies. One week after, filamentous bacteria begin to penetrate these microbial colonies and will form a dense mat of

filamentous bacteria three weeks after teeth were cleaned. Corncob formations are the typical stable structure of mature, supragingival dental plaque and were shown to consist of streptococci adhering to a central axis of long rods,[13, 14] yeast cells or hyphae.[109]

As supragingival plaque increased, inflammatory changes such as swelling and pocket formation occur resulting from a more anaerobic environment. A bacterial shift from Gram-positive coccoid species to a larger proportion of Gram-negative bacteria, motile rods and spirochetes is also observed. The distinctive subgingival microbiota characterized by Gram-negative and anaerobic bacteria becomes established 3-12 weeks after supragingival plaque formation, which can be described as mature biofilm. Many of the subgingival microorganisms are motile and therefore the structural organization of subgingival plaque is different from that seen supragingivally. Bacterial aggregates resembling test-tube brushes characterize mature subgingival plaque.[14] These test-tube brush formations consist of *Tanarella forsythia* and *Fusobacterium nucleatum* arranged perpendicularly around lactobacilli.[109]

When examining natural tooth surfaces in patients with different levels of periodontal health and disease, Listgarten[13] found that the microbial flora associated with periodontal health differs from the flora found in different types of periodontal disease. It is now accepted that the red complex bacteria (*Porphyromonas gingivalis, Tanarella forsythia, Treponema denticola*) described by Socransky et al[110] are highly associated with deeper probing depth and bleeding on probing.

Pyrosequencing technology allows a more comprehensive examination of taxonomically heterogeneous bacterial community. Using this technology, a study compared the bacterial composition of periodontal health and disease.[111] The results confirmed previous observations by

Socransky with regard to the pathogenicity of *P. gingivalis, T. denticola* and *T. forsythia*; however, they found that *T. forsythia* was only the sixteenth most prevalent pathogen in diseased sites while *Filifactor alocis* was as prevalent as the first two. It was also found that community diversity was higher in disease.

Despite this association between certain pathogens and periodontal disease, it is primordial to remember that dental biofilms are dynamic. While some bacteria are structural members of the plaque (*Actinomyces, Fusobacterium, Tannerella*), others colonize the already formed biofilm by co-aggregation (*P. intermedia, P. gingivalis, P. micra*).[109] For example, *P. gingivalis* is a key-stone subgingival pathogenic species, and is now known to be a common inhabitant of the supragingival biofilm through its binding to *S. gordonii*.[112, 113] This highlights the fact that supragingival and subgingival plaque are intimately related as supragingival plaque control results in both quantitative and qualitative changes to the composition of the subgingival plaque. Co-aggregation of bacteria not only facilitates bacterial colonization, but also metabolic communication, and genetic exchange. These interrelations of bacteria growing in biofilms allow the expression of phenotypes that differ from planktonic bacteria and exhibit greater tolerance to antimicrobial agents, oxygen, and alterations in pH.[114, 115]

2.7.2 Around Dental Implant and Healing Abutment

Biofilm colonization and formation on dental implants seems to be similar to that of teeth.[14, 116, 117] Early colonizers species, predominantly streptococci, are found in gingival fluid around dental implants and natural teeth.[118] The late colonizers (*Porphyromonas, Prevotella, Capnocytophaga,* and *Fusobacterium species*) that are the putative periodontal pathogens bind to the early colonizers and form a more complex and mature biofilm.[119, 120] Subgingival plaque at

freshly placed implant and neighbouring teeth analyzed with checkerboard DNA-DNA showed that colonization occurred within 30 minutes after implant placement, and a continual increase in the bacterial load was observed over a 12-week period;[118] however, the early colonization patterns differed between implant and tooth surfaces based on the colonizing bacterial species.[118]

Similarly, a recent study using a broad-range PCR technique to analyze crevicular fluid samples found that certain bacterial genera were present at several teeth sites but not at implant sites.[121] Twenty different genera were identified both at tooth and implant sites. The most frequent genera were *Fusobacterium, Prevotella, Porphyromonas, Streptococcus, Campylobacter,* and *Neisseria*. Nineteen of the 20 genera were identified at teeth, while only seven were at implants. Twelve bacterial genera like *Neisseria* or *Campylobacter* were frequently isolated at dental sites but not found at implant sites, while only one bacterial genus was found solely at implant sites.

Using the pyrosequencing technology, microbial signatures were examined in the following four groups of ten subjects: peri-implantitis, chronic periodontitis, periodontal and peri-implant healthy.[122] The results showed a wide diversity of microorganisms for each groups. Peri-implant communities (healthy and disease implants) contained significantly greater abundance of gram-negative bacteria than subgingival communities. Surprisingly, healthy implants demonstrated the highest levels of gram-negative anaerobes and lowest abundance of gram-positive aerobes when compared with the other groups. The proportion of gram-negative anaerobes was also significantly different between peri-implantitis and periodontitis, being more elevated in the peri-implantitis group. Despite the identification of unsuspected organisms (i.e. high abundance of *S. mutans* and *B. fibrisolvens*), the peri-implant biofilms demonstrated

significantly lower diversity than subgingival biofilms in both health and disease, while the dominant species were not the same in all individuals. Amazingly, the levels of certain genera (*Treponema, Prevotella, Campylobacter* and *Eubacterium*) that are important in the aetiology of periodontitis were significantly higher in peri-implantitis, but also peri-implant health when compared to periodontitis. The authors concluded that the peri-implant microbiomes differ significantly from the periodontal community in both health and disease, and that peri-implantitis is a microbial heterogeneous infection with predominantly gram-negative species, which appears to be less complex than periodontitis.

Also using the pyrosequencing technology, Dabdoub et al[123] compared adjoining peri-implant and periodontal microbiomes in health and disease. They found that almost 85% of the individuals shared less than 8% of abundant species between adjoining tooth and implant. Interestingly, when red complex bacteria (periodontal pathogens) were present in subgingival sulcus, they were found in the peri-implant sulcus in only 37% of the cases. These findings confirmed the previous study that showed significant difference between the peri-implant and periodontal microbiomes even when in close proximity. The "reservoir" theory (periodontal pathogens from one area colonizing an adjacent tooth) should therefore be revisited according to the authors, and future studies should aimed to identify peri-implant pathogens.

In light of the mentioned studies, the differences between the colonizing species around teeth or implants, it was suggested that the surfaces properties of the implants might influence the biofilm composition.

2.7.3 Implant & Abutment Surfaces Topography

Biofilm formation includes four phases, which are bacterial transportation to the surface, initial adhesion, bacterial attachment, colonization and plaque maturation. The surface topography was found to play crucial role in the biofilm formation.

During the adhesion phase, the bacterium come in direct contact with the surface but this interaction is weak and reversible. One of the theories to explain initial adhesion is the thermodynamic approach based on the surface free energy (SFE), where the water film between the interacting surfaces has to be removed. SFE is also called wettability, which makes it obvious that surface with higher wettability are more prone to bacterial adherence. It was reported that hydrophobic surfaces, such as Teflon, harboured 10 times less plaque than hydrophilic surfaces, like enamel over a 9-day period.[124] However, it was also showed that SFE played an effect on plaque maturation on a 3-month-old plaque. Indeed, it was shown that supragingival and subgingival plaque had a higher proportion of cocci and lower proportion of motile organisms and spirochetes on Teflon coated abutment, when compared to titanium abutment.[125]

Although moderately roughness has been associated with better bone integration, roughness has also been associated with higher microbial adhesion and maturation. Quyrinen et al[125] found that abutment with a Ra of 0.8 μm had 25 times more bacteria than a surface with a Ra of 0.3 μm. However, an abutment surface below a Ra of 0.2 μm did not decrease further the bacterial adhesion and is since then suggested as the threshold surface roughness below which bacterial adhesion cannot be further reduced.[126] Bürgers et al[127] compared purely machined and sandblasted and acid-etched titanium specimens. They found that the higher surface roughness (Ra of 0.95 μm) of sandblasted and acid-etched titanium specimens directly influenced the initial

bacterial adhesion, both in vivo and in vitro, when compared to the lower surface roughness of the machined specimens (Ra of 0.15 μm). The higher surface free energy (SFE) of the machined specimens did not seem to be as critical in this study, whereas previous studies showed that higher SFE would facilitate bacterial adhesion.[128, 129] On the other hand, Quirynen and Van Assche[130] found similar biofilms formation around minimally rough implant (turned) and moderately rough implant (TiUnite) during the first year of implant loading. This is not surprising since healing around the implant did occur and subgingival samples were collected around similar abutments.

In summary, initial bacterial adhesion and biofilm maturation will be improved by high SFE, but also by rougher surface that provides protection from shearing forces. However, it seems like the influence of surface roughness is greater than SFE in bacterial adhesion.

2.7.4 Implant & Abutment Fit

The abutment-implant interface offers an ideal niche for bacterial colonization from where it can even leak into the implant screw-hole.[131, 132] It has also been shown that microorganism have the capability to leave, through this interface, the internal part of the implant and seep into the surrounding environment.[133] Recent studies showed that two-piece dental implants harboured a broad spectrum of gram-positive and gram-negative aerobes and anaerobes, especially rods and cocci,[134] but also Candida's species, which are known to be much bigger than bacteria.[133] It was also shown that butt-joint interface implant systems were significantly less resistant to microbial leakage when compared to tube-in-tube connections. When the connections were manually torqued (i.e. torque value lower than recommended by the manufacturer), greater intra-implant microorganisms' colonization was observed in both the butt-joint and tube-in-tube

connections.[133] Such leakage has been associated with area of inflammation at the implant-abutment.[135]

2.8 Dental Implant Decontamination and Disinfection

As it was discussed earlier, a cause and effect relationship between biofilm formation on implants and peri-implant mucositis was demonstrated when using the experimental gingivitis model.[88-90] Similarly, it was shown that mechanical debridement combined with systemic antibiotics and CHX rinse in cases diagnosed with peri-implantitis improves the clinical and microbiological parameters over a 12-month period.[136] From these findings, it can be hypothesized that bacterial biofilm on implant surfaces causes peri-implantitis, and that the removal of these bacteria is the essential part of the treatment. Although peri-implant mucositis lesions are reversible and can usually be controlled with non-surgical treatment,[90, 137-139] the non-surgical treatment of peri-implantitis is not effective and the surgical approach is not predictable.[140-143] This is quite alarming when considering the high prevalence of peri-implantitis reported in the literature.

There are three general surgical interventions in case of peri-implantitis depending of the morphology of the peri-implant defect.[140] The first one consist of an access flap surgery to clean and decontaminate the implant surface where aesthetic is of concerns or in case of shallow defects. The second type of procedures is an apically positioned flap, which can be combined with implantoplasty where the exposed threads of the implant are eliminated and polished, to facilitate professional and home oral hygiene. Finally, where circumferential or intra-bony defect is present, bone regeneration and re-osseointegration therapy should be performed.

An important reason for the poor predictability of such surgical treatment in case of peri-implantitis is the difficulty to decontaminate the implant surface due to its screw shape and roughness of the implant surface. The threads of the implant do not allow proper instrumentation; while the lacunae intended to favour better osseointegration, also provide protection to the bacteria from the much bigger periodontal instrument. Therefore, many clinicians use chemotherapeutics agents, laser and photodynamic therapy or mechanical methods, such as abrasive powder and implantoplasty, to attempt eliminate the biofilm from the implant surface during access surgery. Unfortunately, very limited evidences exist to support one practice over the other. Furthermore, each method has advantages, but also disadvantages.

2.8.1 Mechanical Methods

2.8.1.1 Implantoplasty

This method consists in the removal of the threads and smoothing the implant surface. The aim is to reduce the roughness of the titanium surface to decrease plaque adherence, thus decreasing plaque adherence.[144] Following implantoplasty, recession is to be expected and may compromise the esthetics and increase food impaction. It is also possible that the metal debris remaining in the surrounding tissues might cause foreign body reaction,[145] and the implant core might be at risk of corrosion.[146]

2.8.1.2 Air Powder Abrasive

The use of propelled abrasive powder by a stream of compressed air was found to be effective in cleaning contaminated implant surfaces.[147] However, subcutaneous facial emphysema has been reported with this technic and should be use with extreme caution or even be avoid.[148]

2.8.1.3 Ultrasonic Scaler

Ultrasonic scaler with a metal tip might produce defects in titanium implant surfaces whereas plastic-coated scaler probes cause minimal damage to implant surfaces and have a polishing action but can leave plastic deposits behind on the implant surface.[149] However, another study found that when applied on rough surfaces, ultrasonic scaler with metal tip produced a smoother surface and removed bacteria more efficiently than the ultrasonic scaler covered by a plastic tip.[150]

2.8.1.4 Curette Scaler

Metal curettes were shown to decrease the roughness of implant surfaces and reduce the attachment of the early colonizer *Streptococcus sanguinis*.[151] On the other hand, incomplete removal of biofilm was found with the use of nonmetal curettes on titanium implant surfaces.[145]

2.8.2 Chemical Methods

2.8.2.1 Citric Acid

Citric acid has been widely used in dentistry for its demineralization capacity and for the detoxification of titanium implant surface. Its capacity to remove biofilm on machined titanium surfaces and hydroxyapatite-coated implants has been shown, but not on plasma-sprayed implants.[9] The difference between the hydroxyapatite-coated and plasma-sprayed is thought to be due to the demineralization capacity of the citric acid. The disadvantages of citric acid are the composition changes of the implant surface,[152] and its effect on osteoblasts[153] (decrease proliferation) and fibroblasts[154] (decrease attachment and spreading).

2.8.2.2 Ethylene Diamine Tetraacetic Acid (EDTA)

EDTA is also widely used in dentistry because of its chelating capacity. When compared to citric acid, its main advantage is its neutral pH, but it is less effective to remove biofilm from implant surface.[11]

2.8.2.3 Hydrogen Peroxide (H_20_2)

Hydrogen peroxide is a strong oxidizer, bleaching agent and disinfectant. Similarly to EDTA, it is less effective than citric acid to remove biofilm from titanium surface.[12] When tested on single species biofilm on titanium surface, H_20_2 was found to be solely effective against *Candida albicans*, but not against *Streptococcus sanguinis* or *Staphylococcus epidermis*.[8]

2.8.2.4 Chlorhexidine (CHX)

Chlorexidine is the most frequently used antiseptic in periodontics because of its well-known bactericidal properties. It is not surprising that its ability to decontaminate implant surfaces has been studied. Despite its bactericidal and substantivity capacity, CHX failed to provide better detoxification than other technics.[9, 12] Furthermore, CHX can affect the osteoblasts viability in a dose and time dependent manners[155] and inhibit cell proliferation and collagen synthesis.[156]

2.8.2.5 Tetracycline

Tetracycline HCL is a bacteriostatic antibiotic with substantivity capacity.[157] However, Tetracycline HCL paste has not been shown to be effective in removing biofilms from titanium implant surfaces.[12]

2.8.2.6 Saline

Most of the cited experimental studies that reported some efficacy of chemotherapeutic agents in removing biofilm or decreasing its viability did use sterile water or saline solution as a control. Interestingly, a clinical trial using sterile saline to irrigate copiously the implant surface and rubbed its surface with surgical gauze soaked in sterile saline was an effective anti-infective therapy of peri-implantitis when combined with adjunctive systemic antibiotics, and the clinical results were maintained after 12 months.[158]

2.8.3 Laser and Photodynamic Therapy

Laser and photodynamic therapy are advertised with many advantageous characteristics, such as easy handling, hemostatic effects, effective calculus ablation, or bactericidal effects against periodonto-pathogens. Such benefits could potentially improve the detoxification of titanium implant surface.

2.8.3.1 CO_2 Laser

CO2 laser has been shown to burn contaminants on the implants surfaces but failed to remove them completely under dry conditions and only partially under wet conditions.[152] The viability of adhered *S. sanguinis* and *P. gingivalis* on SLA titanium surface was reduced with CO_2 laser, but similar results were achieved with a 30 seconds exposure to CHX.[159]

2.8.3.2 Er:Yag Laser

An in vitro study showed that when used at low-energy densities, the erbium-doped yttrium-aluminum-garnet (Er:YAG) laser had a high bactericidal potential on common implant surfaces without causing morphologic changes of the implant surface or inducing excessive heat.[160] An animal study found that Er:YAG laser treatment can debride the implant surface

effectively when compared with plastic scaler and new bone formation was observed on the laser-treated implant surface.[161] However, two recent meta-analyses of randomized controlled trials couldn't find evidence of superior effectiveness when compared to non-surgical or surgical mechanical debridement with or without chemotherapeutic agents, or air abrasive.[162, 163]

2.8.3.3 Photodynamic Therapy (PDT)

Photodynamic therapy is based on the application of photosensitive dyes activated by a light with a specific wavelength, which transform oxygen into ions and radicals that are highly reactive and kill the microorganisms.[144] However, the dyes do not differentiate between bacteria and host cells, thus damage to the surrounding tissues can't be fully avoided.[164] Despite the rational of PDT, a clinical trial comparing mechanical debridement with or without PDT did not show significant differences in the clinical parameters between the two groups.[165]

2.8.4 In Vivo Studies

Most studies reported in the literature with regard to the treatment of peri-implantitis are case reports or case series, which present different treatment protocols developed by practitioners. One of the most cited case series is the one from Leonhardt et al.[166] The long-term outcome (5-year follow-up) of access surgery was evaluated in nine subjects with 26 implants affected by severe peri-implantitis. Surgical protocol included access flap mechanical debridement combined with 10% hydrogen peroxide rinse, followed by two weeks of 0.2% CHX rinse twice per day and systemic antibiotic. Seven implants were lost in four individuals, four implants continued to lose bone, while resolution of inflammation and preservation of the supporting bone was observed in 19 of the 26 implants with peri-implantitis (58%). However, a more recent retrospective study of 245 patients surgically treated for peri-implantitis showed that

it was not feasible to arrest the progression of the disease in 54.7% over a period ranging between nine months to 13 years.[167]

Very few in vivo studies compared different implant surface decontamination approaches, most of them are of very short-term and even fewer assess microbiological outcomes.

A recent double-blinded randomized clinical trial was conducted to measure the effect of implant surface decontamination with CHX and cetylpyridinium chloride (CPC) on microbiological and clinical parameters over a 12-month period.[168] Resective surgery consisting of apically positioned flap with bone recontouring combined and implant surface debridement and decontamination with a solution of 0.12% CHX and 0.05% cetylpyridinium chloride (CPC) was performed on 15 patients with peri-implantitis. The same treatment protocol was performed on another 15 patients with peri-implantitis but a placebo solution was used to decontaminate the implant surfaces. Both treatments resulted in significant bacterial load reduction, but the implant decontamination with CHX and CPC showed greater immediate suppression of anaerobic bacteria on the implant surface. However, no difference was observed in the clinical parameters. Similarly, when comparing the above solution to 2% CHX solution during the same type of procedure, no clinical, radiographic of microbiological difference could be observed in 44 patients with peri-implantitis equally and randomly assigned to both groups.[169]

The potential benefit of a CO_2 laser in the treatment of peri-implantitis was studied in 32 patients with 73 ailing implants. The implants were air-polished after the granulation tissue was removed from the defects. Thirty-nine implants were also decontaminated with CO_2 laser. There were no significant differences in the distance from implant shoulder to the first bone contact

between implants treated with or without the laser four months after the surgery.[170]

A similar study compared the clinical and radiographic parameters after implant decontamination by Er:YAG laser or plastic curettes debridement followed by swabbing with cotton pellets soaked in saline during regeneration surgery.[171] Thirty-two patients suffering from advanced peri-implantitis were treated with flap surgery, implantoplasty at buccally and supra-crestally exposed implant followed by the randomly assigned surface decontamination protocol. The intra-bony component was then augmented with a xenogeneic bone mineral and covered with a collagen membrane. Clinical and radiographic parameters recorded at baseline and after 6 months of non-submerged healing showed comparable radiographic bone fill and improvements of the clinical parameters without significant differences between both groups.

In summary, it seems like the different decontamination protocols of the infected implant surface, leads to very similar clinical and radiographic outcomes in vivo. Moreover, it seems like saline rinse achieves just as good results to laser, air-abrasive or chemical disinfection. Therefore, it can be speculated that other factors than implant decontamination will affect the treatment outcomes. These factors will not be discussed in this thesis, but could include systemic conditions, smoking habit, oral hygiene, etc.

2.8.5 In Vitro Studies

Despite the poor predictability of the surgical protocols, there is still sufficient consensus that the mechanical removal of biofilm from the implant surface should be supplemented by chemical decontamination during access surgery,[172] or any other decontamination protocol (laser, photodynamic therapy, etc.). Only a few in vitro studies have assessed the efficacy of biofilm removal from titanium substrata similar to dental implants.

Zablotsky et al[12], studied the decontamination effect of ten treatment modalities on contaminated grit-blasted titanium strip with radioactive LPS from *Escherichia coli*. They found that 30 seconds of air-powder abrasive was the only treatment modality able to remove significantly greater amounts of LPS from the titanium surfaces when compared with burnishing the surface with a cotton pellet dipped in sterile water for one minute. Stannous fluoride (1.64%), CHX (0.12%), and Tetracycline HCL did not remove significantly more LPS when compared to untreated strip. Burnishing the surface for one minute with cotton pellet dipped in sterile water, or hydrogen peroxide (H_2O_2 3%), or chloramine T (1%), or citric acid (pH1), or scaling the surface with a plastic sonic instrument did remove significantly more LPS than untreated strips. However, no statistical differences were observed between these modalities.

Similarly, Dennison et al[9] studied the decontamination effect of distilled deionized water for one minute, CHX (0.12%), citric acid and air-powder abrasive system for one minute. Three types of implant were used: machined, plasma-sprayed and hydroxyapatite coated. All implants were previously coated with iodinated LPS from *Porphyromonas gingivalis*, which would allow measurement of radioimmunoassay as an outcome measure. Air abrasive treatment was the most effective of the four treatments on plasma-sprayed implants, was equally as effective as citric acid on hydroxyapatite-coated implants, and was equally as effective as water or CHX on machined implants.

Mouhyi et al[173] compared the atomic composition of non-contaminated and contaminated titanium foils surfaces, which were characterized by X-ray photoelectron spectroscopy. They found that a combination of citric acid, H_2O_2 and carbon dioxide laser irradiation provided similar atomic composition to non-contaminated surfaces. None of the chemotherapeutic agents,

neither the laser would provide similar atomic composition of the non-contaminated titanium foils. The authors concluded that the proposed treatment protocol could be effective in cleaning the contaminated titanium surface. It is noteworthy to mention that the substrata used in this study were similar to the Branemark implant, and the titanium foils were attached to complete dentures and worn intra-orally for 24 hours without any oral hygiene.

Chin et al[174] assessed the antimicrobial efficacy of CHX 0.2% and sodium fluoride 0.055% on five different orthodontics anchorage devices. Four were made of titanium whereas the fifth one was made of stainless steel. Overnight biofilm growth was used to contaminate the substrata. They found that biofilm formation was governed by roughness of the implant surface, that the chemotherapeutic agents used in this study did not remove biofilm but contained significantly less viable organisms than untreated micro-implants.

Ntrouka et al[11] assessed the effectiveness of the different chemotherapeutic agents on biofilm-contaminated titanium surfaces (Straumann disks: SLA and machined surfaces). Their first experiment consisted in the treatment of *Streptococcus mutans* biofilms with either five minutes of EDTA, citric acid, cetylpyridium chloride, H_2O_2, Ardox-X followed by citric acid, H_2O_2 followed by citric acid, CHX, or water. *S. Mutans* was selected for the first experiment because of its potential to form highly adhesive biofilms. Citric acid, H_2O_2 and Ardox-X were the most potent agents in killing *S. mutans* biofilms. The authors also assessed the biofilm removal capacity by isolating proteins from the *S. mutans* biofilms that remained on the discs after treatment with the three most potent chemotherapeutic agents (citric acid, H_2O_2 and Ardox-X). Citric acid and H_2O_2 removed significantly more protein in the *S. mutans* biofilm than water. The second experiment consisted in the treatment of polymicrobial biofilms grown for 48 hours,

which were treated by citric acid, H_2O_2, Ardox-X, Ardox-X followed by citric acid or H_2O_2 followed by citric acid. CHX was used as positive control and water as negative control. Citric acid was the most effective treatment, killing 99.9% more bacteria compared with water, and was also statistically significantly more effective than CHX. The combination of citric acid with H_2O_2 or Ardox-X did not produce any added effect. Since both surfaces of the discs (SLA and machined) were exposed to the biofilms, treated and analyzed at the same time, it is not possible to apply the results to one or the other surface type.

Gosau et al[10] investigated the anti-adherence activity and the bactericidal effect of six different disinfection solutions on polymicrobial biofilms grown for 12 hours intra-orally on machined titanium discs fixed on acrylic oral appliance. The specimens were disinfected for one minute with one of the following solution: NaClO 1%, H_2O_2 3%, CHX 0.2%, citric acid 40%, Plax (triclosan), or Listerine. All tested solutions significantly reduced the total number of attached bacteria. With the exception of Plax and citric acid, all the tested solutions were also capable of inactivating attached bacterial cells compared with the control.

Burgers et al[8] studied the effect of the same six antimicrobial agents on single-species biofilms of *Streptococcus sanguinis, Staphylococcus epidermidis,* and *Candida albicans* on titanium implant surfaces. Bioluminometric assay was used to quantify the remaining vital fungi, whereas fluorescence microscopy was used to quantify the bacterial load and viability. The specimens were incubated for two hours on machined pure titanium discs and were rinsed for one minute in NaClO 1%, H_2O_2 3%, CHX 0.2%, citric acid 40%, Plax (triclosan), Listerine or sterile saline as control. The results showed that only NaClO was effective against the three microorganisms tested. CHX 0.2% was effective against *S. sanguinis* and *C. albicans*.

A recent study assessed the ability of photodynamic therapy to decrease the bacterial load on Nobel TiUnite implant surface.[175] The new and sterile implants were placed in black plate and contaminated with saliva from a patient diagnosed with peri-implantitis. The implants were kept in saliva for five minutes. The implants were then treated for five minutes with 0.12% CHX gluconate or for five minutes of photodynamic therapy. Both treatments decreased significantly bacterial load when compared to the non-treated implants, but no difference was noted between the treatments.

Cold atmospheric plasma was also tested to remove non-mature biofilms formed in situ for 24h and for 72h on sandblasted and etched titanium surfaces commonly used for dental implants.[176] A combination of plasma treatment and non-abrasive air-water spray caused inactivation of the biofilm and a significant reduction of protein counts as measure by contact culture, confocal laser scanning microscopy and colomitrecally quantification. Total removal of the non-mature biofilm was also possible with further plasma jet treatment as explored with SEM imaging. However, a significant increase of the titanium discs temperature was observed with a mean 43.2^{o}C +/- 4.9^{o}C when using plasma jet power of 5W. The temperature decreased to 39.1^{o}C +/- 3.1^{o}C in the center of the plasma jet when using plasma jet power of 3 W.

Low direct current on non-mature multispecies biofilms grown on SLA titanium discs also showed promising results.[177] A direct current of 10 mA applied for 10 minutes detached more biofilms from the implant when used as the cathode, but no viable bacteria could be recovered if the disc was used as the anode, as estimated by colony-forming unit and confocal laser scanning microscopy. However, there are still some concerns with regard to clinical application.

All the decontamination protocols used in the above-mentioned studies were tested on non-mature polymicrobial biofilms and / or on single specie biofilms. None of the proposed biofilm models can pretend to mimic a mature polymicrobial in vivo biofilms. Furthermore, many of these studies used substrata similar to machined titanium discs, which may be less biofilms adherents than the moderately rough implants used these days.[125, 127] Because biofilms are much more complex than a single microorganism and more resistant to chemotherapeutics agents,[15, 16] more studies are needed to assess the effect these antimicrobial agents, or any other decontamination technics, in the treatment of peri-implantitis on a moderately rough titanium surface substrata commonly used in dental office.

Chapter 3: Aims & Hypothesis

3.1 Aims

The aims of this study are to develop a biofilm model on implant SLA surface that closely mimics in vivo biofilm and to determine its susceptibility to antimicrobial agents compared to saline rinse. A new chlorhexidine decontamination agent formula will also be developed for a pilot study, as well as a new potential decontamination technique (peeling-off technique).

3.2 Hypotheses

The following hypotheses were tested:

I. The use of the decontamination agents will remove biofilm from the SLA dental implant surfaces more significantly than the saline rinse;

II. The use of the new chlorhexidine decontamination agent will be more effective than the others chemical agents at removing biofilm from SLA dental implant surfaces; and

III. The peeling-off technique will be more effective than the chemical agents and saline rinse at removing biofilm from the SLA dental implant surfaces.

The null hypothesis is that the addition of decontamination agent or peeling-off technique will not remove mature multispecies bacterial biofilms from SLA dental implant surfaces more efficiently than saline rinse.

Chapter 4: Materials & Methods

4.1 SLA Implant Surface Biofilm Model

The experiment was repeated three times in a triplicate format. Each experiment consisted in 18 sterile SLA discs (5 mm diameter by 1mm thickness; Straumann®, Basel, Switzerland), which were used as the biofilm substrate. All SLA discs were coated with bovine dermal type I collagen (10 μg/mL collagen in 0.012M HCl in water; Cohesion, Palo Alto, CA). The coating process was done by overnight incubation at 4^{o}C in the wells of a 24-well tissue culture plate containing 2 ml of the selected solution. After the overnight incubation, the 18 SLA discs were rinsed in 2 ml of sterile phosphate-buffered saline (Sigma-Aldrich, Saint Louis, MO) and then placed in the wells of a 24-well tissue culture plate containing 2 ml of dispersed dental plaque in brain heart infusion broth (Becton-Dickinson, Sparks, MD) with a minimum bacterial cell concentration of $3.2X10^{7}$ CFU/ml. The subgingival dental plaque was collected from three healthy volunteers (one donor per experiment). The discs were incubated under anaerobic conditions (AnaeroGen; Oxoid, UK) at 37^{o}C for 21 days and the medium was changed every 7 days.[15-17]

4.2 Treatment Protocol

SLA disks with established mature biofilms were subjected to 2 minutes treatment with chemical agents routinely used by clinicians treating peri-implantitis. Only one time point was used that reflect practical application of these agents in a clinical setting (during surgery). In order to eliminate the paste or gel from the implant surface, it was determined by pre-testing experiment that six increments of 1 ml 0.9% Sodium Chloride Irrigation USP (0.9% NaCl; Braun, Irvine, CA) was requested to remove the chemical agent from the SLA disk surface. Each

increments of 1ml rinse was applied with a manual Fisherbrand Finnpipette II (100 µl to 1000 µl; Fisherbrand, Pittsburgh, PA) producing a constant flow similar to a rinse in a clinical setting using a Monoject syringe.

For application, disks were first rinsed in the 2 ml well with six increments of 1 ml 0.9% NaCl to remove the established biofilm leaving a cleaned visual SLA implant surface. The following chemical agents were then applied on triplicate disk for 2 minutes: chlorhexidine 1% in methylcellulose 3% gel (CHX group), 35% phosphoric acid gel (Etch group; Ultra-Etch, Ultradent, South Jordan, UT), and tetracycline paste (Tetra group: 250 mg tetracycline tablet mixed with 0.9% NaCl to make a thick paste). After the chemical treatment, the agent was removed with six increments of 1 ml 0.9% NaCl. To control for the mechanical effect of rinsing, three samples per experiment were rinsed with six increments of 1 ml 0.9% NaCl (Rinse group), and three samples per experiment were rinsed with twelve increments of 1 ml 0.9% NaCl (Double Rinse group) without any of them being subjected to chemical agents. Three disks per experiment were not rinsed or treated with chemical agent and served as control. Disks were then prepared for the assessment of efficacy of biofilm removal (SEM) and killing (CLSM).

4.3 Scanning Electron Microscopy (SEM)

Each disk was then transferred into a well containing 1 ml of 0.1M PIPES (pH 7.4) (Sigma-Aldrich, Saint Louis, MO) for 2 minutes. Proteins were then fixed by placing each disk into well containing 1 ml of 2.5 % glutaraldehyde in 0.1M PIPES (pH 7.4) for 30 minutes. Each disk was rinsed again in 1 ml of 0.1M PIPES (pH 7.4) for 5 minutes. Following that, the lipids were fixed in 1 ml of 1% osmium tetroxide in 0.1M PIPES (pH 6.8) for 60 minutes. The disks were washed with 2 ml of double distilled water 3 times for 5 minutes to completely remove the

osmium solution. 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%.

Following dehydration, the disks were placed in 1 ml of 100 % hexamethydilazane (HMDS; Sigma-Aldrich, Saint Louis, MO) 3 times for 5 minutes to dry the samples. Each disk was then attached to metallic stub using electrical conducting double-sided adhesive, and the contour of each disk/stud was painted with colloidal silver to improve the electrical conductivity. Samples were coated with 8 nm of iridium using the Leica EM MED020 Coating System (Leica Microsystems, Wetzlar, Germany).

Each sample was examined using scanning electron microscopy (Helios NanoLab 650 Focussed Ion Beam SEM, Oregon, USA). Images of the center of each disk were taken at a voltage of 1 kV at various magnifications providing an overview image with a 99.5 μm X 71.45 μm field of view representing a surface area of 7109.275 μm^2, and a detailed image with a 29.8 μm X 21.4 μm field of view representing a surface area of 637.72 μm^2. These fields of view correspond roughly to a magnification of 1500 and 5000 times respectively. Using ImageJ 1.47v software (National Institutes of Health, Bethesda, MD), a bacterial cell count was performed for each 5000-magnification image.

Figure 1: Samples ready to be examined under SEM (control SLA disk covered by biofilm on the left side, rinsed SLA disk on the right side).

4.4 Confocal Laser Scanning Microscopy (CLSM) Pilot Study

During a set of experiments, one extra SLA disk sample per group were prepared, rinsed or treated as above but were observed under confocal laser scanning microscopy after staining the samples for live/dead bacteria using LIVE/DEAD BacLight Bacterial viability kit L-7012 (Molecular Probes, Eugene, OR). A x10 lens was used to observed the mounted specimens. Each disk was scanned in three randomly selected areas of the biofilm. Fluorescence from stained cell was viewed by using a CLSM (Nikon Eclipse C1; Nikon Canada, Mississauga, ON, Canada). Simultaneous dual-channel imaging was used to display green and red fluorescence. CLSM images of the biofilms were acquired by the EZ-C1 v. 3.40 build 691 software (Nikon) at a field resolution of 512 by 512 pixels and a pixel resolution of 2.5 μm, giving the scanned field area of 1.69 mm^2. Confocal images of ≈ 50 slices were captured from the top to the bottom of the biofilm (each slice was 0.5 μm thick). [15-17]

The surface areas occupied by live or dead bacteria were calculated with Imaris 7.3.0v software (BITPLANE, South Windsor, CT). The volume ratio of green fluorescence (live bacteria) to green-and-red fluorescence (live and dead bacteria) was calculated in three randomly selected fields, indicating the percentage of live bacteria in the remaining biofilms.

4.5 New Chemical Agent and Peeling-off Effect Pilot Study

Following the same protocol described from section 4.1 to 4.3, a new chemical agent was tested on three samples. This chemical agent consisted of a mix of cetrimide 0.3%, chlorhexidine gluconate 0.1% and EDTA 0.5% (pH 7.0) in methylcellulose 3% gel (C.C.E. Group).

Similarly, the bacterial mechanical peeling effect of Flexitime Xtreme, a vinylpolysiloxane precision impression material (VPS; Heraeus Kulzer, Hanau, Germany) was assessed on three samples prepared following the same steps. Each disk were rinsed with six increments of 1 ml 0.9% NaCl, impressed with the VPS material, which was peeled off after it has set. Disks were rinsed again with six increments of 1 ml 0.9% NaCl before being prepared for SEM analysis. This peeling effect was tested following the observation of the ability of this material to remove incorporated grit from the SLA surface. For this pilot study, three samples rinsed with six increments and three samples rinsed with twelve increments of 1 ml 0.9% NaCl were used as control.

Figure 2: Samples impressed with VPS.

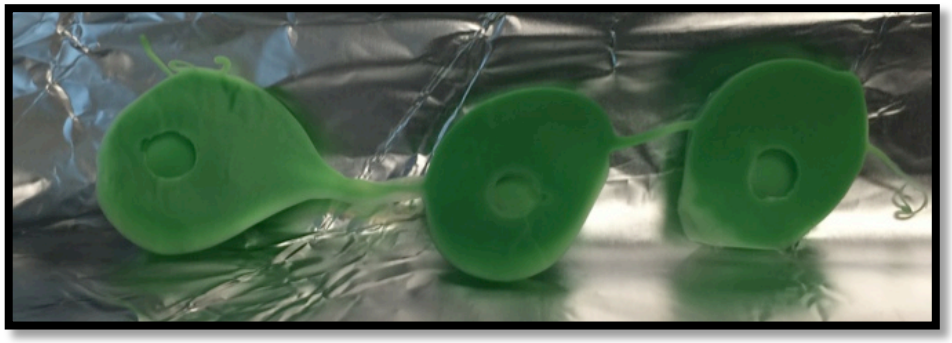

Figure 3: Impression material after peeling the SLA disks.

4.6 Statistical Analyses

The distribution analyses of the results were completed with the Kolmogorov-Smirnov Goodness-of-Fit Test (SPSS version 16.0; SPSS Inc., Chicago, IL). In consequence, the results of all the SEM experiments were analyzed by using one-way analysis of variance (ANOVA) test with Tuckey post hoc analysis at a significance level of $p < 0.05$ (SPSS version 16.0; SPSS Inc., Chicago, IL), whereas the results of the CLSM experiments were analyzed by using Kruskal-Wallis Test with Tamhane post hoc analysis at a significance level of $p < 0.05$ (SPSS version 16.0; SPSS Inc., Chicago, IL).

Chapter 5: Results

5.1 SLA Implant Surface Biofilm Model Results

After three weeks, the biofilm thickness reached about 25 µm and showed the presence of multitude of rod and coccoid shape organisms. In areas of artificial separation, SLA surface is visible with numerous coccoid organisms present in the SLA surface lacunae (Figure 4). Dividing cells and clusters of bacteria that are closely packed and interlaced by a well-developed inter-microbial matrix are observed in this biofilm model (Figure 5). Coccoid cells surrounding filamentous organisms suggesting of corncob formations are also seen. These characteristics are typical of natural structural network of mature biofilms.

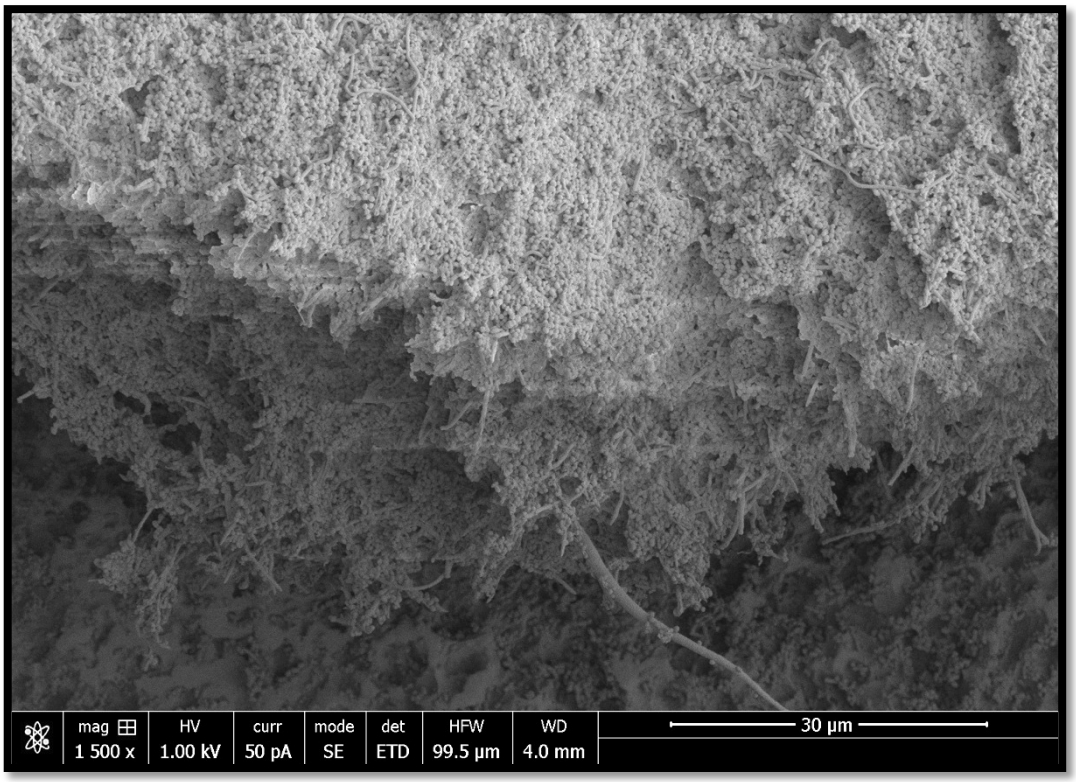

Figure 4: A scanning electron micrograph of 3-week-old biofilms on SLA implant surface.

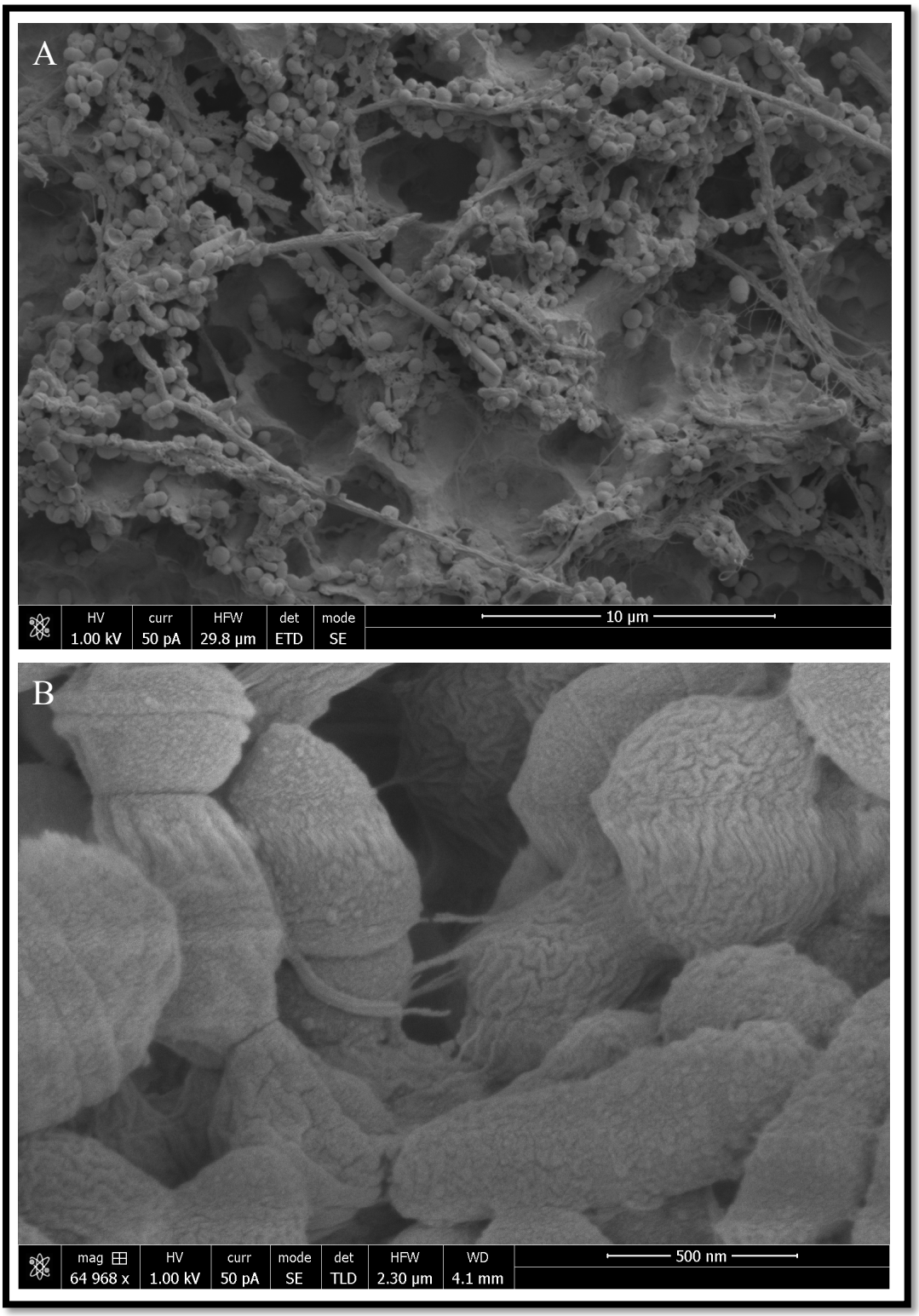

Figure 5: A scanning electron micrograph of 3-week-old biofilms on SLA implant surface. Inter-microbial matrix and bacterial clusters (A). Close interaction between cells through filaments (B).

5.2 Main Study Results (SEM Analysis)

A total of 45 disks were viewed under SEM (excluding 9 disks in the control group), providing the same number of images from which bacteria counts were performed using ImageJ64. Most of the mature biofilm was easily removed (Figure 8), leaving only a very small proportion of bacteria on the SLA titanium surface after the first set of saline rinse. The mean bacteria count per unit area, representing the viewed surface of 637.72 μm^2, was 469.6 for the Rinse group, 292.8 for the Double Rinse group, 313.7 for CHX group, 347.2 for the Etch group, and 310.3 for the Tetra group (Table 1). The Kolmogorov-Smirnov distribution test showed the results of this study were normally distributed (p=0.200). Therefore a One-way ANOVA with post hoc Tuckey test was used for the statistical analysis. When comparing the groups, only the Double Rinse group removes significantly more bacteria than the Rinse group (p=0.047). However, there was no significant difference between the Double Rinse group and the three chemical agents groups, which were all rinsed with a total of 12 increments of 1 ml 0.9% NaCl (Figure 6).

When comparing the percentage of bacteria remaining on the disks using the Rinse group as control (100% remaining), the Double Rinse group removes 37.6% more bacteria, CHX group removes 33.2% more bacteria, Etch group removes 26.1% more bacteria and Tetra group removes 33.9% more bacteria (Table 1 and Figure 7). The differences were statistically significant for the Double Rinse group (p=0.009), the CHX group (p=0.028) and the Tetra group (p=0.027). There was no significant difference between the Double Rinse group and disinfectant groups. A SEM image for each treatment protocol is shown in Figure 8. Figure 9 present the same images of Figure 8 after bacterial counts using Imagej64 (except Figure 9A).

Main Study	Rinse (SEM)	Double Rinse (SEM)	CHX (SEM)	Etch (SEM)	Tetra (SEM)
Bacteria / unit area	469.6 (56.8)	292.8 (41.3)	313.7 (39.6)	347.2 (34.2)	310.3 (41.5)
% of Rinse	100 (6.6)	62.4 (1.0)	66.8 (0.7)	73.9 (2.2)	66.1 (2.1)

Table 1: Mean bacteria count per unit area and percentage of remaining bacteria (main study).

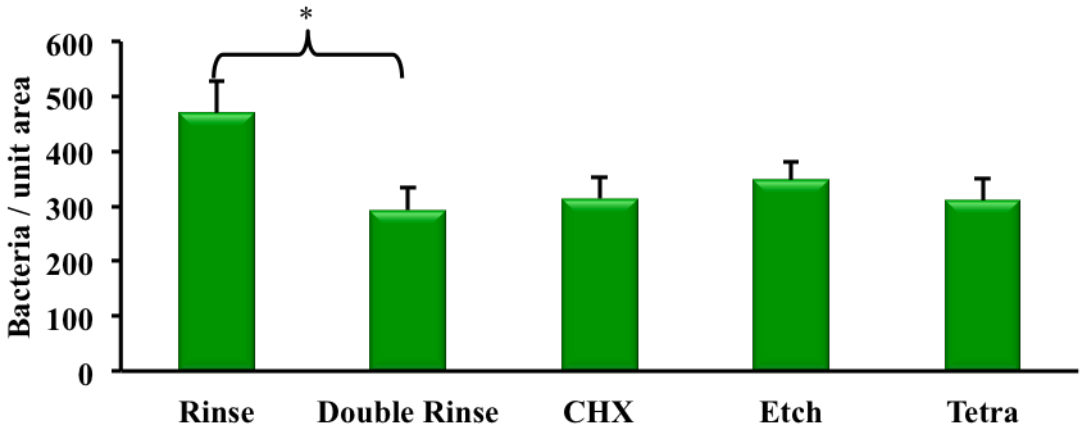

Figure 6: Mean bacterial count per unit area. One-way ANOVA with post hoc Tuckey test, *p<0.05 (main study).

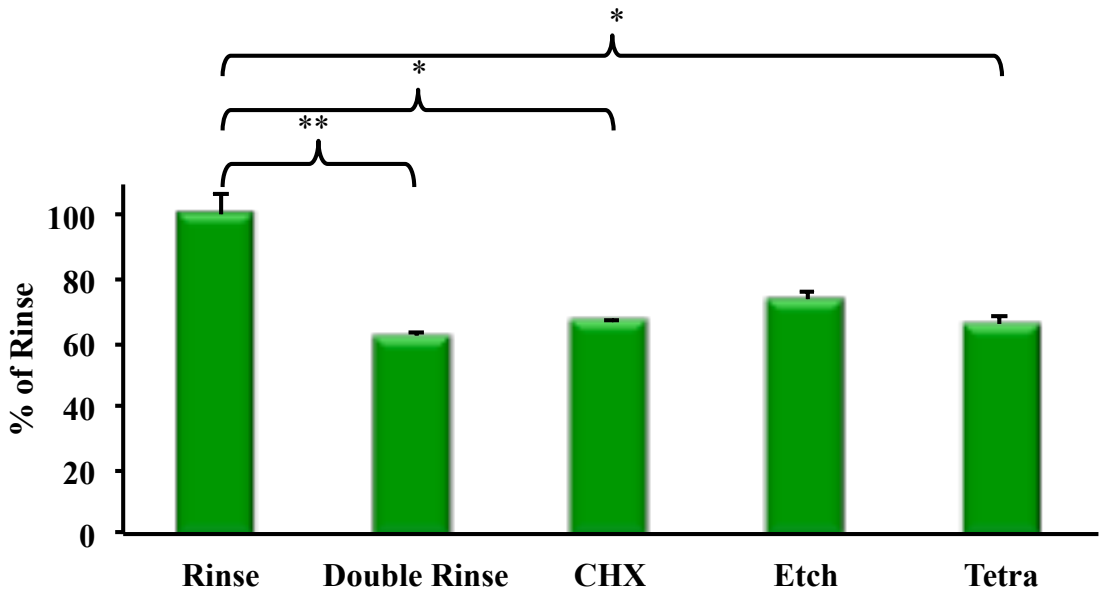

Figure 7: Remaining bacteria expressed in % relative to rinse group as control. One-way ANOVA with post hoc Tuckey test, *p<0.05, **p<0.01 (main study).

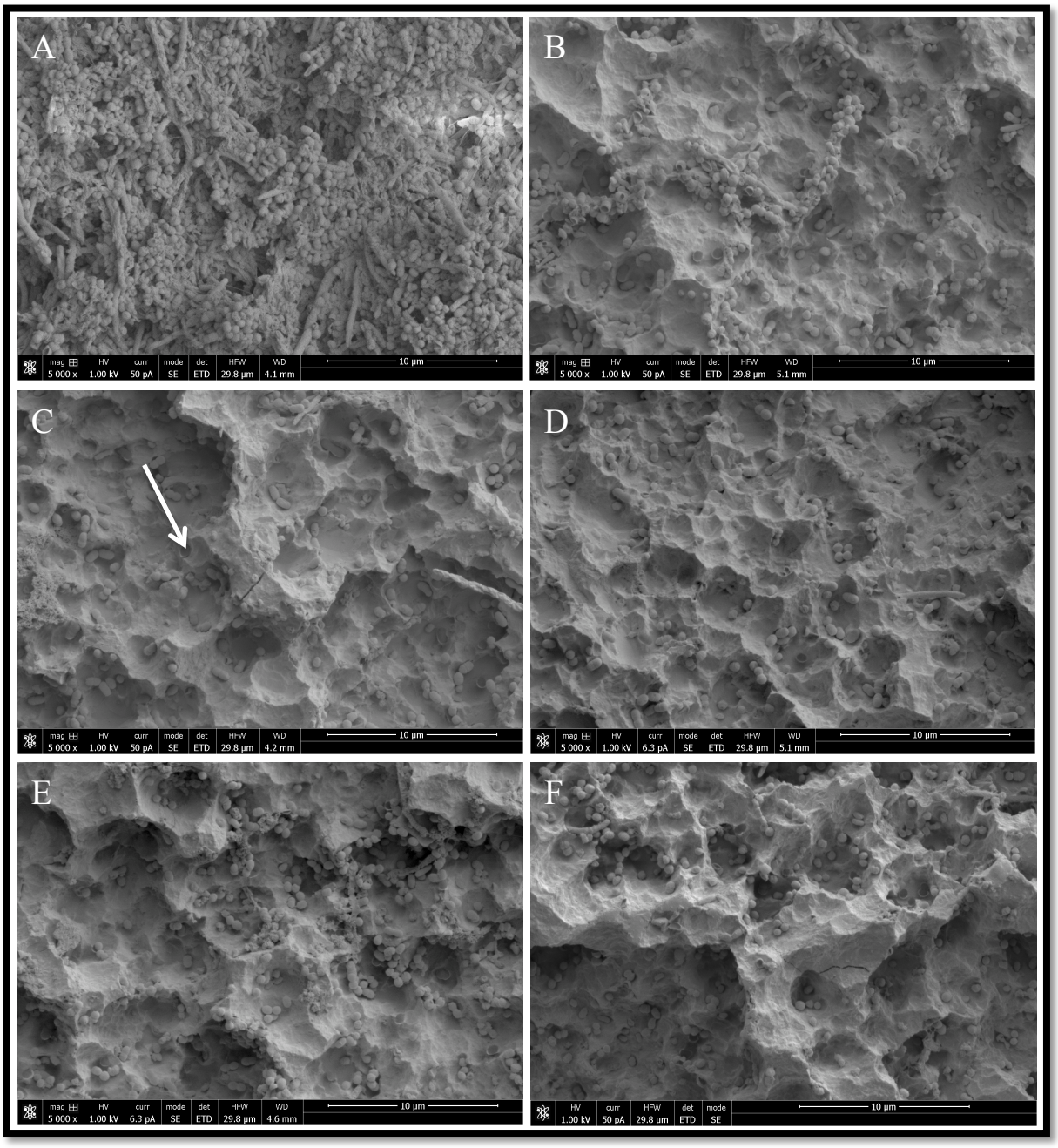

Figure 8: SEM images at 5000X of 3-week-old SLA biofilms after treatment with disinfecting agents. Control biofilm (A), SLA disk treated with physiological saline (B: rinse and C: double rinse), 1% CHX gel (D), 35% phosphoric acid gel (E), and tetracycline paste (F). The white arrow point a ruptured bacterial cell still attached to the titanium surface.

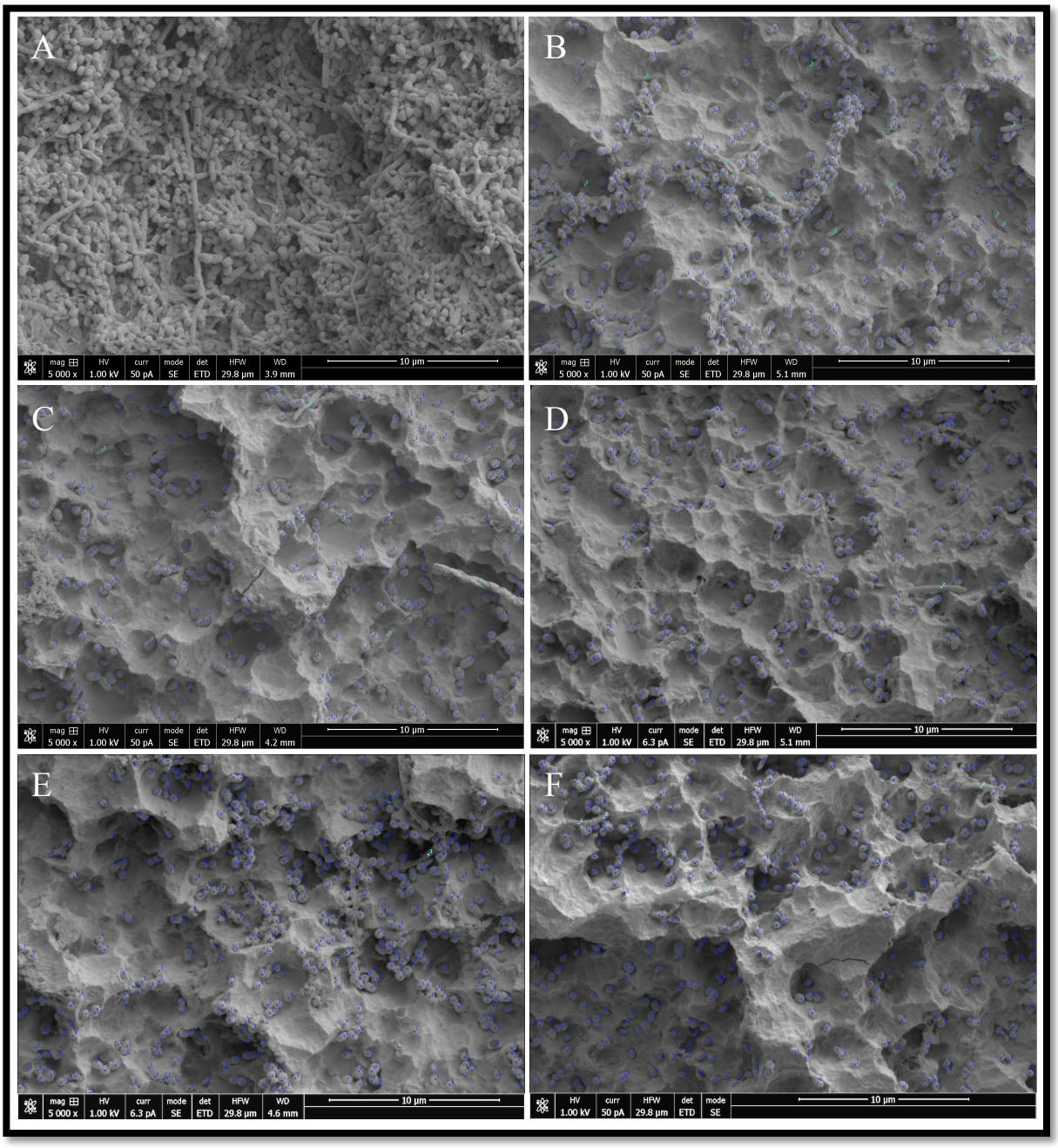

Figure 9: Bacterial count using ImageJ64. Control SLA biofilm (A). SLA disk treated with physiological saline (B: rinse and C: double rinse), 1% CHX gel (D), 35% phosphoric acid gel (E), and tetracycline paste (F).

5.3 CLSM Pilot Study Results

One disk per group was viewed under CLSM for a total of 6 disks. From each disk, three randomly areas were used to assess the percentage of live and dead cells. The Kolmogorov-Smirnov distribution test showed the results of this study were not normally distributed ($p<0.001$). Therefore, Kruskal-Wallis Test with Tamhane post hoc analysis was used for the statistical analysis. The Control biofilm thickness reached approximately 29.5 μm. The Control, Rinse and Double Rinse disks showed insignificant percentage of dead cells with ratio of live to total cells of 99.6%, 99.6% and 99.4% respectively (Table 2, Figure 10 and 11). The CHX and Etch groups showed a significant reduction of viable bacteria within the biofilms compared with the Control, Rinse and Double Rinse disks. The proportion of killed cells in the CHX and Etch was 11.8% ($p=0.023$) and 6.9% ($p=0.017$) respectively. The Tetra group had a proportion of dead cells of 3.9%, which was not significantly different from all other groups.

Pilot Study	Control (SEM)	Rinse (SEM)	Double Rinse (SEM)	CHX (SEM)	Etch (SEM)	Tetra (SEM)
% Live to Total	99.6 (0.2)	99.6 (0.1)	99.4 (0.4)	88.2 (4.1)	93.1 (2.2)	96.1 (1.7)

Table 2: Proportion of the live cell volume of the entire biofilm volume in the SLA biofilm (pilot study).

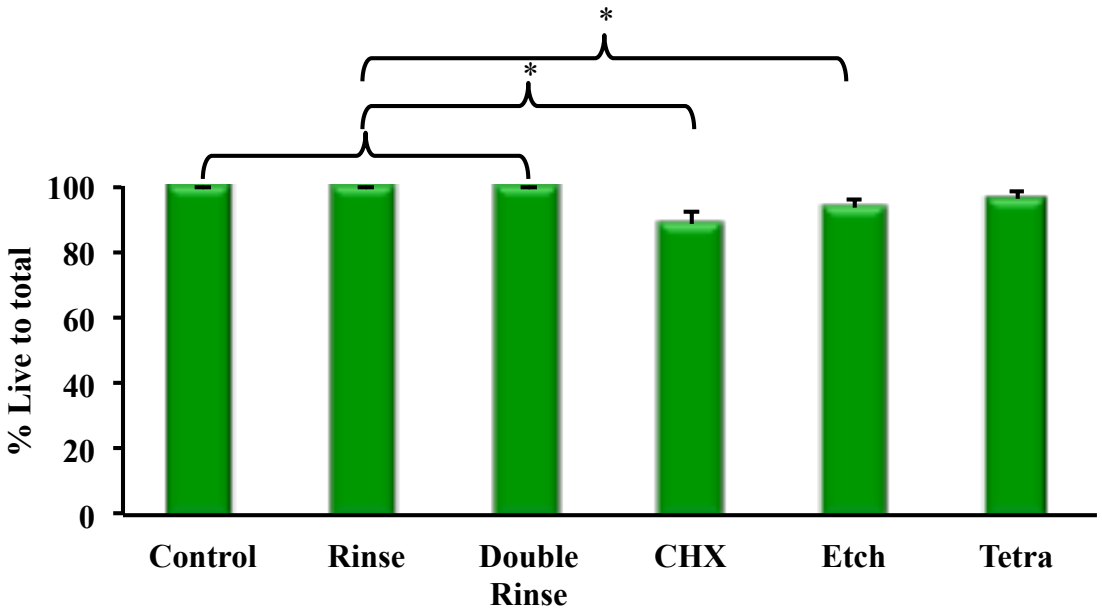

Figure 10: Ratio of live to total cells (%). Kruskal-Wallis Test with Tamhane post hoc test, *p<0.05

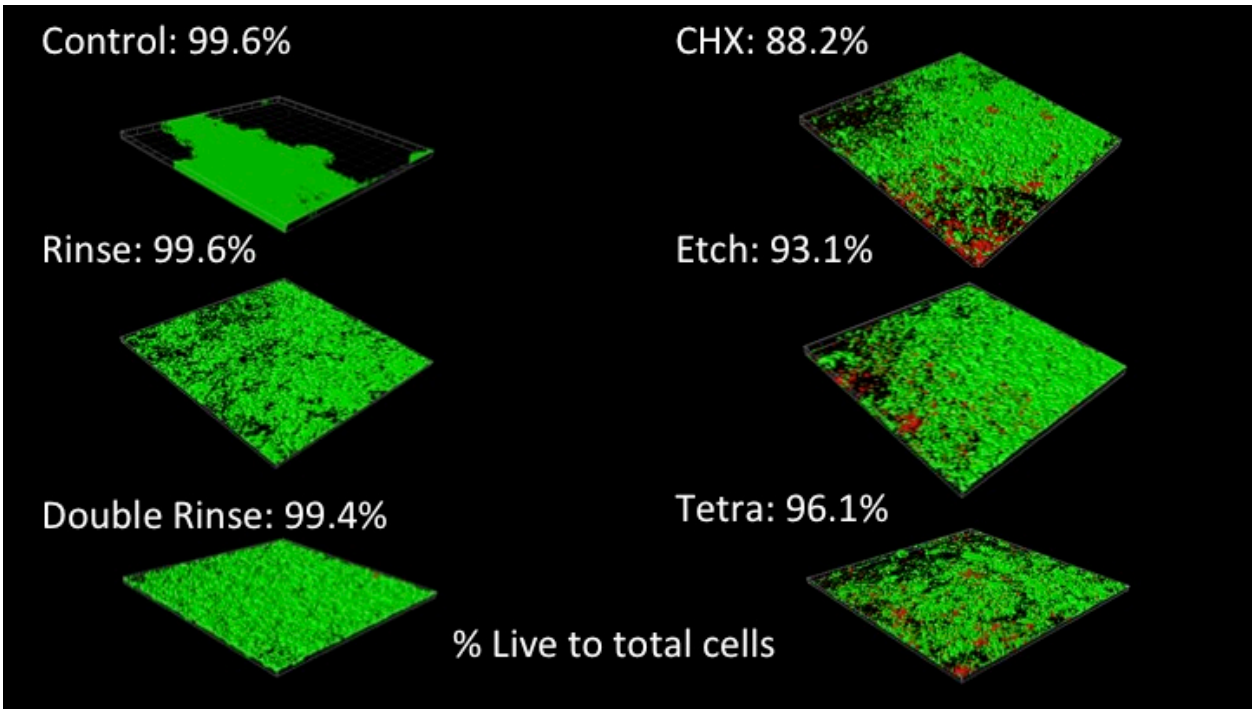

Figure 11: Three-dimensional constructions of CLSM scans of 3-week-old SLA biofilms after treatment with disinfecting agents and viability staining. Control biofilm, biofilm treated with physiological saline (rinse and double rinse), 1% CHX gel, 35% phosphoric acid gel, and tetracycline paste. Green (viable cell) and red (dead cells).

5.4 New Chemical and Peeling-off Effect Pilot Study Results

Three disks per group provided one image each for a total of 12 images for this study. All images were taken at the center of the disk as per the main study. The Kolmogorov-Smirnov distribution test showed the results of this study were normally distributed (p=0.002). Therefore a One-way ANOVA with post hoc Tuckey test was used for the statistical analysis. The results of this pilot study are presented in Table 3 and Figure 12. The mean bacteria count per unit area, representing the viewed surface of 637.72 μm^2, was 606.3 for the Rinse group, 539.3 for the Double Rinse group, 535.7 for C.C.E. group, and 622.7 for the Peeling group. There was no significant difference between any groups.

When counting the ratio of total cells remaining using the mean bacterial count of the Rinse group as denominator, the percentage of bacteria remaining on the disks was 88.9% for the Double Rinse group, 88.3% for the C.C.E. group, and 102.7% for the Peeling group. The Double Rinse group removes 11.1% more bacteria; C.C.E. group removes 11.7% more bacteria; whereas the Peeling group leaved 2.7% more bacteria on the disks surface when compared to the Rinse group (Table 3 and Figure 13). No statistically significant difference was noted between the groups for this study. A SEM image for each treatment protocol is shown in Figure 14. Images of Figure 15 are the same presented in Figure 14 after bacterial counts using Imagej64. Figure 16 shows the inside of the VPS material that was in contact with one of the contaminated implant. Particles can be seen on the VPS surface, which seems to be grits but could also be bacteria cell (small round shapes).

Pilot Study	Rinse (SEM)	Double Rinse (SEM)	C.C.E. (SEM)	Peeling (SEM)
Bacteria / unit area	606.3 (41.1)	539.3 (71.5)	535.7 (40.5)	622.7 (88.4)
% of Rinse	100 (6.8)	88.9 (11.8)	88.3 (6.7)	102.7 (14.6)

Table 3: Mean bacteria count per unit area and percentage of remaining bacteria (pilot study).

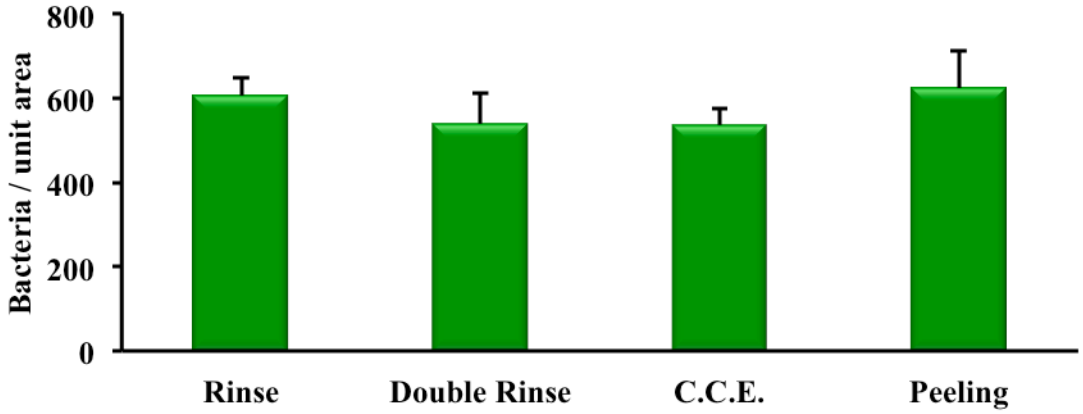

Figure 12: Mean bacterial count per unit area (pilot study).

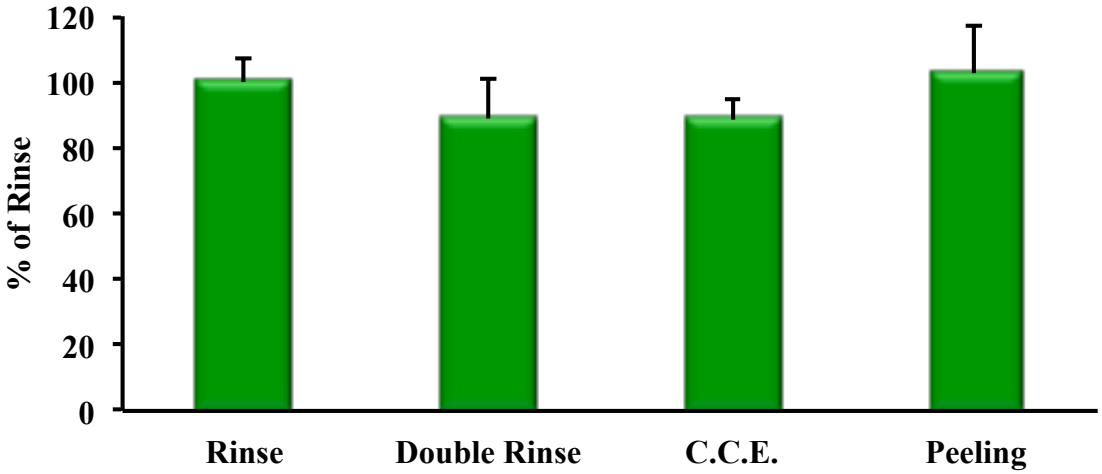

Figure 13: Remaining bacteria expressed in % relative to rinse group as control (pilot study).

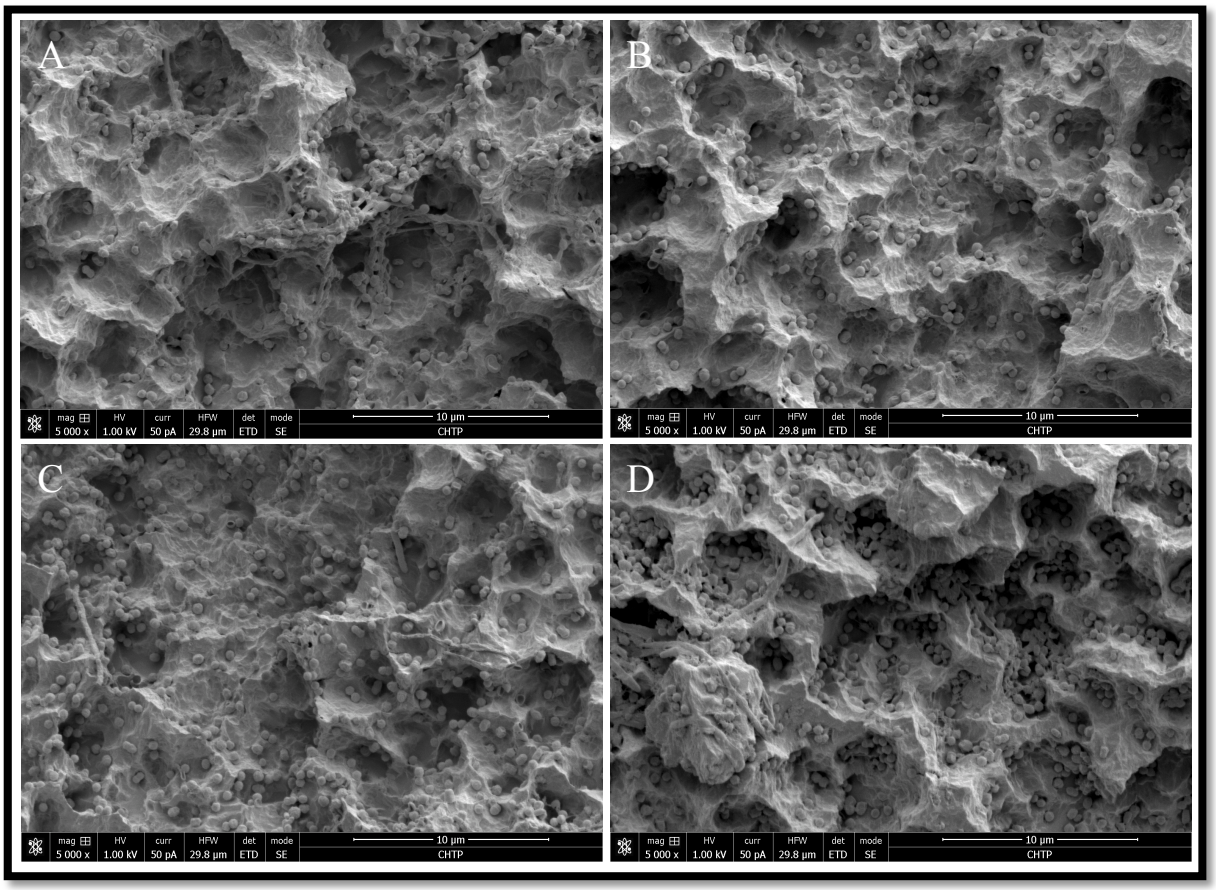

Figure 14: SEM images at 5000X of 3-week-old SLA biofilms after treatment with disinfecting agents. SLA disk treated with physiological saline (A: rinse and B: double rinse), C.C.E. gel (C), peeling (D).

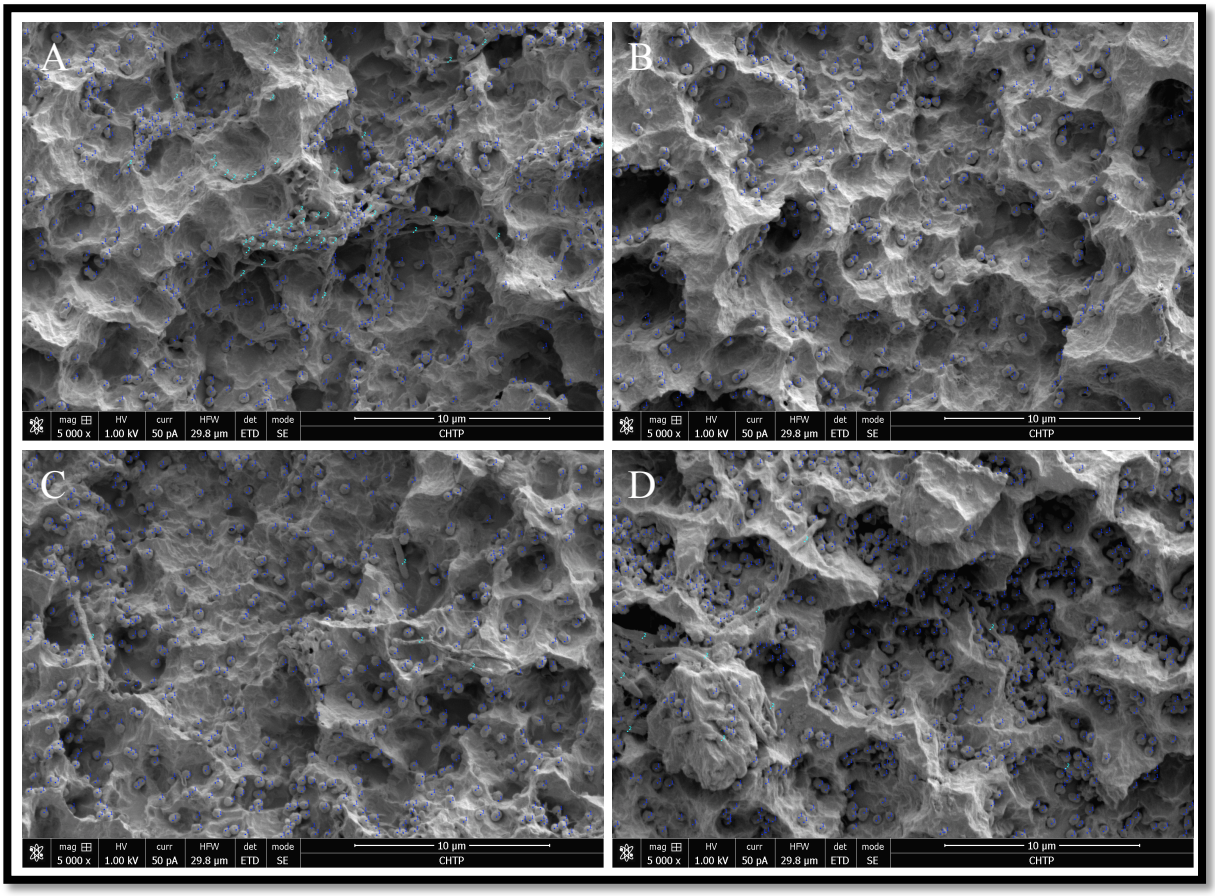

Figure 15: Bacterial count with ImageJ64. SLA disk treated with physiological saline (A: rinse and B: double rinse), C.C.E. gel (C), peeling (D).

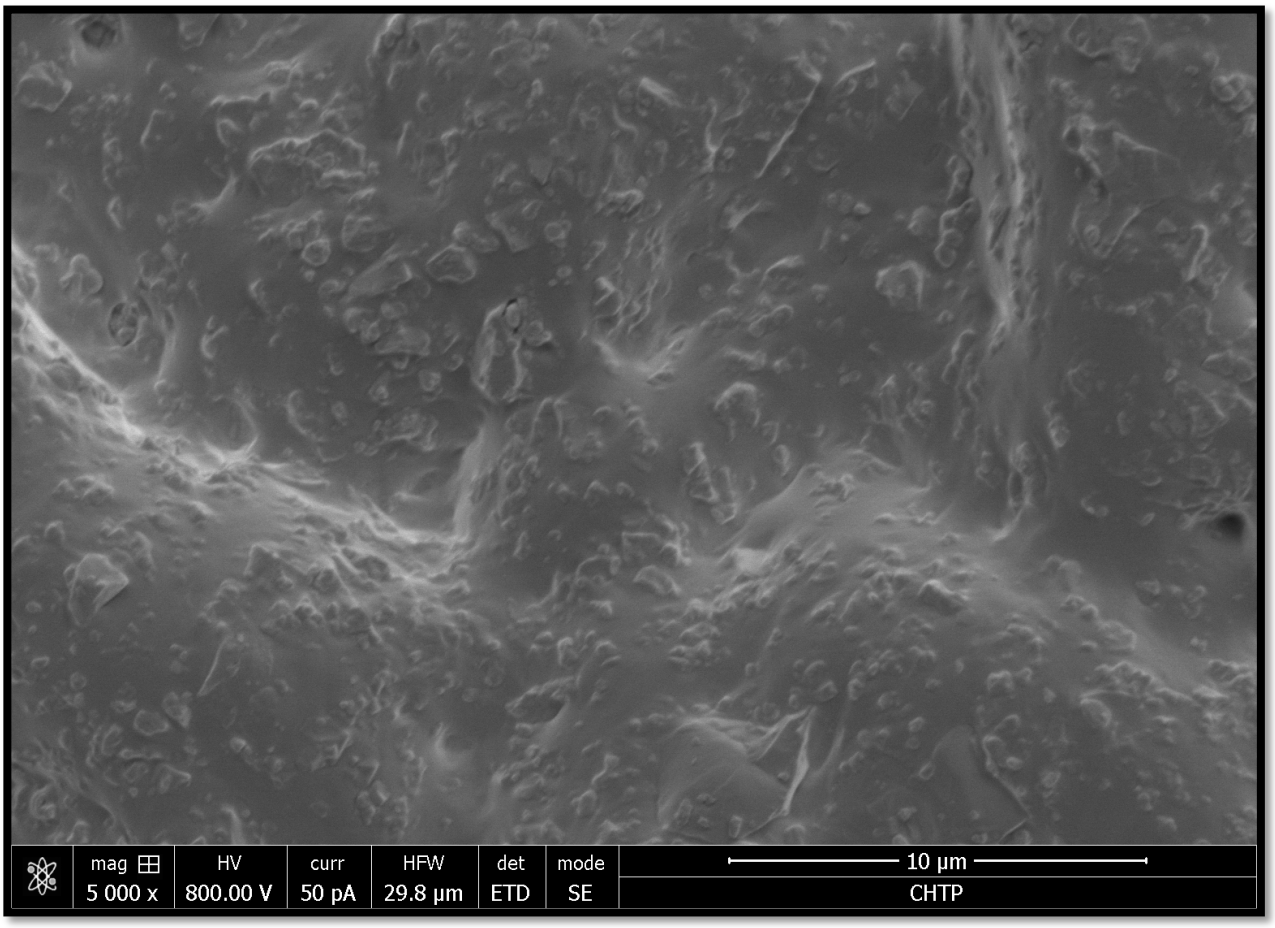

Figure 16: Image of the vinylpolysiloxane material that was in contact with the contaminated implant.

Chapter 6: Discussion

Dental implants supported suprastructures have become the gold standard for replacing missing teeth in partially and completely edentulous patients. While survival and success rates of dental implants have been reported to be high, biological complications are of increasing concerns. Indeed, there are limited scientific data on the treatment of peri-implant diseases, especially with regard to peri-implantitis. Furthermore, the unpredictable results of such treatment aiming at controlling the infection point out weakness of our understanding of biofilm interaction at the surface of the dental implants.

As of the writing of this thesis, we are not aware of any studies that have assessed chemotherapeutic agents on multispecies and mature biofilms (three weeks old), mimicking in vivo biofilms, on SLA commercially pure titanium surface.

6.1 Mature and Multispecies Biofilms Model

The titanium disc used in the present study were coated with type I collagen prior to be exposed to dental plaque in BHI. This is because the organic part of the bone matrix is mainly composed of type I collagen, it can be expected that demineralized bone around implant would leave type I collagen remnant at the surface of the implant in case of peri-implantitis. Furthermore, some bacteria adhesins have affinity to type I collagen. It is known that *S. gordonii* and *S. mutans* cells exhibit binding to collagen and a growth response to collagen is mediated by adhesins.[178] As discussed previously, *P. gingivalis* is a key pathogen for both periodontitis and peri-implantitis, which is highly associated with *S. gordonii*.

The SEM images of the control biofilms obtained in this study clearly show that multispecies and mature biofilms can be grown on SLA titanium surface. The dividing cells, the well-developed inter-microbial matrix, and the closed adaptation of the cells within each other and the SLA titanium surface are supporting this affirmation. The CLSM results also showed that over 99% of the three-week-old control biofilms were viable. This particular finding is a strong indicator that our biofilms model on SLA titanium surface has the potential to serve as multispecies mature biofilms for future peri-implantitis in vitro studies.

The thickness of the control biofilms observed under SEM (approximately 25 μm) or CLSM (approximately 29.5 μm) is however narrower than what other studies reported. Using the same biofilm growing protocol on hydroxyapatite collagen coated discs, Shen et al[17] showed that the thickness of the biofilms reached 155 μm after three weeks of anaerobic incubation. This is most likely caused by the different substrata used.[179] However, another study found that biofilms reached a mean thickness of 91.2 μm on sand blasted and acid etched titanium discs after only 72 hours of intra-oral exposure.[176] This difference is quite surprising considering the short exposure to in situ biofilm in this study. The condition of incubation, anaerobic vs. aerobic could have played a role in plaque accumulation. Indeed, biofilms in the latest study were grown in situ and collected on an oral appliance, which had to be aerobic. In the present study, the anaerobic environment in which the biofilms were grown could have potentially limits its growth.

In order to maintain the biofilms at the surface of the titanium discs during SEM samples preparation, hexamethydilazane (HMDS) was used as a drying agent instead of the critical point dryer (CPD). This technique is commonly used to dry samples prior imaging

them under SEM to decrease the chance for distortion in cell morphology and minimizes artefacts. This technique involves passaging liquid CO_2 across the specimen at a certain temperature and high pressure, which could alter or remove the biofilms from the surface of the titanium disks. On the other hand, HMDS drying allow specimens to be dried without being exposed to high pressure or changes in temperature. Previous studies showed similar results in the quality of the images when comparing the HMDS drying technique to the CPD technique.[180, 181] One difficulty arise however with this technique as it left a small residual film in the center of some samples, which prevented taking the image at the center of the discs. The image was therefore taken as close as possible to the center but where the titanium discs surface could be clearly visualized. This problem was not reported in the previously cited articles.

Nonetheless, three weeks anaerobic incubation provided viable biofilms that are more likely to mimic in vivo biofilm associated with peri-implantitis. Indeed, it was shown in a recent study that peri-implant biofilms had the higher level of gram-negative anaerobes bacteria, both around healthy and diseased implants.[122]

6.2 Biofilms Removal or Decontamination Effect

Ideally, complete biofilm removal should be achieved during access flap surgery in case of peri-implantitis, which refer to decontamination. According to Mosby's Dental Dictionary, second edition, decontamination is the process of making a person, object, or environment free of microorganisms, radioactivity, or other contaminants.

The results presented here undoubtedly illustrate that the chemotherapeutic agents used in this study do not remove bacteria from the surface of the SLA titanium discs more effectively

than the sterile sodium chloride rinse. This finding supports previous case series and clinical trials that showed no difference in the clinical parameters between disinfectant solutions and sterile saline rinse after the treatment of peri-implantitis cases.[168, 171, 182]

It is actually quite interesting to notice that the double rinse group is the only group to remove significantly more bacteria from the SLA discs when compared to the single rinse group, despite the same quantity of 0.9% NaCl rinse was used for all groups, with the exception of the single rinse group. It is thought that the mechanical effect of the saline rinse was the most important factor in biofilm removal. It can also be speculated that the thickness of the different gels or paste that were applied on the SLA titanium discs to decontaminate the surfaces acted as a barrier, preventing the mechanical removal effect of the rinse.

Another interesting result was that during the pilot study, the double rinse group and new chemical agent (C.C.E. Group) did not remove similar percentage of bacteria when compared with the main study results. From the main study, the double rinse group removed 37.6% more bacteria than the rinse group. Similar percentages were obtained for the three disinfectant agents, ranging form 26% to 34% more bacteria removal. In the pilot study, the double rinse group removed 11.1% and the C.C.E. group removed 11.7% more bacteria than did the rinse group. These results could potentially be explained by the fewer number of samples used for this pilot study. It can also be argued that the biofilms obtained might have been thicker, making it possibly more resistant to removal. However, no control biofilm disc was used for this particular pilot study. Nevertheless, the relationship between the double rinse group and the chemical agent groups were similar within each experiments, suggesting once again that the mechanical removal effect of the rinse is the main factor contributing to biofilm removal.

Orthopaedic medicine also used titanium in the fabrication of joint replacement prosthesis. As per dental implant, biofilms on orthopaedic prosthesis are of concerns. A very interesting study conducted by Bundy et al[183] using jet impingement technique, showed that bacterial adhesion (*S. aureus*) to metal was much higher than fibroblasts. Furthermore, it was found that bacterial detachment is caused more by pressure and that bacterial adhesion to metals is more affected by flow rate. On the other hand, fibroblasts are more affected by shear forces and time.

A previous study from the same group of researchers found that under the influence of jet impingement forces, bacterial cells attached to metals detach by rupturing of cell membranes.[184] This phenomenon could possibly explain what appear to be fragments of bacterial membranes remaining on titanium surface, which was seen on many occasions during the present study (e.g. white arrow on Fig. 8C). This is suggesting that the adhesions of the bacteria to the substrata are actually stronger than their cell wall structure.

To the best of our knowledge, this study is also the first one to quantify bacteria removal with SEM imaging after chemical decontamination of the SLA titanium surface. Although the images are spectacular and prove that complete biofilms removal cannot be achieved with commonly decontamination protocols, this technique also comes with limitations. Indeed, it cannot be determine if the bacteria left in the lacunae of the SLA discs are alive or dead. Furthermore, it is extremely hard, if not impossible, to quantify the total amount of biofilms remaining on the titanium surfaces, i.e. including the inter-microbial matrix. Attempts were made using ImageJ software to quantify the percentage of the surface occupy by the remaining biofilms with shade of gray or shapes analysis. However, it was not possible to differentiate

between the remaining biofilms and the SLA surfaces. These difficulties geared us toward another tool to assess the viability of the biofilms, namely CLSM.

6.3 Disinfection Effect

If it is not possible to completely remove the biofilms from the surface of the discs, can the biofilms be inactivated? The process of destroying pathogenic organisms or rendering them inert is defined as disinfection according to the Mosby's Dental Dictionary, second edition. CLSM has been used in many studies to quantify and describe the viability of the biofilms, and the main advantage of the CLSM is its ability to analyze multi layers of images providing a 3 dimensions view of the biofilms.[10, 15-17, 176, 185]

The results of this CLSM pilot study are somewhat disappointing. Indeed, the best disinfection was provided by 1% CHX gel with a ratio dead to total of 11.8%. The least efficient disinfectant agent used by Gosau et al[10] was Plax with a ratio dead to total ranging from 16% to 39%, while the most efficient was Hydrogen Peroxide ranging from 43% to 92% after chemical agents were applied for one minute on 12-hour biofilms on machined pure titanium. The same study reported results ranging from 41% to 94% for 0.2% CHX. The difference between that study and the results of the present study can be explained by the maturity of our biofilm. Indeed, it has been clearly shown that multispecies biofilms become more resistant to antimicrobial agent as the biofilms mature. This increase in resistance is marked for the three-week-old biofilms and remains similar for even older biofilms.[15, 16] Interestingly, this "three-week mark" coincide with the formation of the distinctive subgingival microbiota characterized by Gram-negative and anaerobic bacteria, which becomes established 3-12 weeks after supragingival plaque formation, as described by Listgarten.[14]

When three-week-old multispecies biofilm on collagen coated hydroxyapatite discs are exposed to 2% CHX, Shen et al[17] found means ratio of dead to total ranging from 19% to 31% for one and three minutes exposition respectively. The difference between that study and the results of the present investigation resides in the application protocol of the chemotherapeutic agents. For the present study, the SLA discs were rinsed first, eliminating most of the biofilms of the samples surfaces. This step was included in our protocol to mimic similar procedure that would be applied during surgical access surgery to clean implant surface prior to the application of the disinfectant agents. On the other hand, previously cited studies applied the chemical agents directly on the biofilms covering the substrata. Therefore, it can be speculated that a greater quantity of biofilms was exposed to the antimicrobial agents. In the present study, the first sets of saline rinse removed all eye-visible biofilm from the surface of the SLA titanium discs. The remaining bacteria after the first rinse were adhering into the deep titanium lacunae. It can be hypothesized that the wettability capacity of the different chemical agents may play a crucial role in the decontamination / disinfection of any moderately rough titanium surface; i.e. if the agent cannot reach the biofilm into the lacunae, it cannot be effective.

6.4 Wettability Effect

Wettability is defined as the angle at which a droplet of liquid interfaces with a horizontal surface; the shape of the droplet varies depending on the type of liquid and surface, thereby influencing the contact angle and thus the wettability (Mosby's Dental Dictionary, second edition). It is known that wettability is improved by high energetic surfaces of the substrata and low surface tension of the wetting liquids. However, the wettability of dental implant is not fully understood and many theorems have been proposed. According to the Wenzel theorem, wetting

liquid completely fills a rough surface topography, including all indentations and pores.[186] However, it is known that after roughening titanium by blasting and etching treatments, the contact angles as been recorded to be as high as 125° on SLA titanium surface[187] but was even recorded to approximate 160^{o} in other titanium surfaces which was blasted with large grits and acid etched with a combination of acids exhibiting almost super-hydrophobicity.[188] This really obtuse contact angle is thought to be caused by entrapped air in the deep lacunae of the SLA titanium surface. This wetting theorem is known as Cassie-Baxter theorem.[186] More recently, it was shown that even if most implant systems commercially available were hydrophobic during the first immersion of the implants in the liquid, a shift to total wettability was observed for all implant systems when repeatedly immersed in the liquid. The de-wetting process can explain this shift from a hydrophobic state toward a more hydrophilic state; a receding contact angle of 0^{o} was observed on all implant surfaces indicating that the adhesion between the liquid and the solid is greater than the cohesive strength of the liquid, therefore leaving a trail of liquid on the implant surfaces.[187, 189] This being said, when placing an implant, the initial wetting of the dry and sterile SLA titanium surface is most likely to follow the Cassie-Baxter theorem.

What are the wettability processes after the implant has been wet, exposed to biofilms, and treated with chemical agents during access flap surgery in case of peri-implantitis? It can be hypothesized that once exposed to a stagnant chemical agents, as it was the case in this study, some regions of the surface present full agent penetration into the topography (Wenzel) and others show partial or no penetration in the deep lacunae due to entrapped biofilms, water, or even air after being exposed (Cassie-Baxter). Following the principle that implant are first hydrophobic and shift toward a hydrophilic state with successive immersion in liquid, it can also

be hypothesized that continuous rinse with the chemical agents could enhance the wettability of the implant surfaces and therefore get in closer proximity to the biofilms.

In this study, three gels and one paste were used with different surface tensions and capillary action, which affect their wettability capacities. The different chemical agents also had different viscosity properties. Viscosity is the ability or inability of a fluid solution to flow easily. High viscosity indicates a slow-flowing fluid (Mosby's Dental Dictionary, second edition). In other words, it is a measure of the consistency of a fluid and its resistance to flow. In a recent study, it was found that surface wettability and liquid viscosity influence the wetting dynamics of individual droplets spreading on solid surfaces; the inertial wetting time is longer for relatively high viscosity liquid; and viscous wetting regime only occur on surfaces with an equilibrium contact angle smaller than a critical angle, which is depending on viscosity of the liquid.[190] The substrata and liquids used in the present study are completely different than those used in the last cited study. Nevertheless, more attention should be placed towards this phenomenon in the choice of the ideal chemical agent to remove/kill biofilms on dental implants. This last cited study also points out the enhanced effect of time. Better results in our disinfection protocol could maybe have been achieved with longer chemical agents exposure.

In summary, the results of the pilot study using CLSM seems to show that biofilms lying in the deep lacunae of SLA titanium discs are minimally affected by the two minutes exposure of 1% CHX gel, 35% UltraEtch, or Tetracycline paste. More studies comparing different chemotherapeutics agents with different viscosity and wettability capacity should be undertaken. Caution is also advisable when analyzing the presented data since only three randomly chosen field of view were observed under CLSM for every single sample per groups.

6.5 Chemotherapeutic Agents

The choice of the chemotherapeutic agents for this study was mainly based on their availability in the general dental practice. As presented in chapter two, these chemotherapeutics agents have been reported to be more or less efficient in the treatment of peri-implant diseases. But none of them were tested on mature multispecies SLA titanium surfaces.

Topical application of Tetracycline HCL has been used as a root conditioner to remove smear layer. This demineralization of radicular dentin to uncover and to widen the orifices of dentin tubules and to expose the dentin collagen matrix before regeneration or soft tissue grafting is achieved by its acidic pH.[191] Tetracycline HCL is a bacteriostatic antibiotic, which was shown to be absorbed by exposed dentin, and was subsequently desorbed over a period of up to 14 days.[157] With this information in mind, clinicians started to use Tetracycline HCL paste to decontaminate implant surface, despite the difference in the substrata (natural tooth vs. titanium). The results of the present study clearly demonstrate topical application of Tetracycline HCL paste was not effective to remove or killed mature and multispecies biofilms when applied for two minutes, which is in agreement with Zablotsky et al.[12]

It has been demonstrated that phosphoric acid use to etch dental tooth structure during direct composite restoration has antibacterial effect.[192, 193] Phosphoric acid at concentration of 2.5% was also shown to be bactericidal against *E. faecalis*.[194] Its antimicrobial activity is thought to be related to an increased external hydrogen-ion concentration, which inhibits many microorganisms' metabolism and consequently their growth.[194] Phosphoric acid is also used in combination with high current density or potential to increase the roughness of implants by anodization in order to facilitate osseointegration.[195] The phosphoric acid used in the present

study, Ultra-Etch from Ultradent, is also marketed as having a proprietary surfactant that allows it to make better contact with tooth structure. However, no information on its interaction with titanium could be found in the literature. Nevertheless, better results of this chemical agent at 35% concentration were expected in the decontamination and disinfection of the SLA titanium surfaces during the present study.

Only one study assessed the clinical and microbiological outcomes of phosphoric acid application around dental implants in a split-mouth design.[6] In this study, all patients were edentulous with maxillary complete removable denture, and mandibular overdenture supported by a bar device on 4 implants. They were all included in a maintenance program, which consisted of traditional mechanical debridement on the control side and intra-sulcular application of 35% phosphoric acid for one minute on the test side. Both test and control implants showed improvement in clinical parameters and reduction of colony-forming units (CFU), but the reduction in gingival index and CFU was significantly larger in the test group. Interestingly, this study was conducted in a population not affected by peri-implant diseases, thus limiting the validity of it.

Ethylenediaminetetraacetic acid (EDTA) is a pH neutral acid widely used in endodontic and periodontal therapy to remove smear layer.[196] It is a chelating agent, which is the ability to sequester metals ions and to diminish their reactivity. However, its antibacterial effect is limited against *E. faecalis*.[194]

There is no doubt that tetracycline HCL paste, phosphoric acid and EDTA can remove smear layer on dentin. However, smear layer on the surface of a natural tooth creates somewhat relatively smooth surfaces, which most likely improve the wettability of the surface. On the other

hand, it can be speculated that the wettability of the SLA titanium surface by the tested chemical agents might be limited by the Cassie-Baxter phenomenon.

CHX is well known antiseptic, which is a cationic bisbiguanide. It has bacteriostatic action at low concentration, but also has bactericidal action at higher concentration.[197] Its bacteriostatic action is due to the alteration of the osmotic balance of the bacteria cell, while its bactericidal action is by cytolysis of the cell. CHX is active against Gram-positive and Gram-negative bacteria (although weaker) as well as fungi and yeasts. The substantivity of CHX is an advantage; its antibacterial effect has been shown to last for 8 to 12 hours. These characteristics may explain why better results in killing the biofilms were achieved with CHX in the present study, despite poor bacterial removal. Three in vitro studies also showed the poor biofilms removal effect of this chemotherapeutics agent.[9, 12, 173]

Cetrimide is an antiseptic consisting of different quaternary ammonium salts, including cetrimonium bromide. The later is a cationic surfactant that reduces the surface tension of liquids, improving their entry into places of difficult access.[198] It was also shown to weaken the biofilm's cohesive forces, disrupting the inter-microbial matrix responsible for biofilms mechanical stability.[199] Furthermore, when mixed to CHX, they have a synergistic activity and have been shown to kill *E. faecalis*, associated with persistent root canal infection, faster and at smaller concentration than chlorhexidine alone.[200, 201]

The combination of CHX, cetrimide and EDTA was thought to provide an effective chemotherapeutic agent to reach in the deepest lacunae of the SLA titanium surface (EDTA), to weaken the biofilm and facilitate its removal (cetrimide), while killing the remaining bacteria

(CHX). Even though this mixture was only tried on three samples, the results of the present study clearly show the limitations of the chemical decontamination and disinfection of SLA titanium.

6.6 Peeling Effect on Mature and Multispecies Biofilms Model

Mechanical peeling using a low viscosity vinylpolysiloxane (VPS) impression material has been used with success to clean dry implant surfaces from debris and grits. From this premise, it was hypothesized that bacteria and biofilms could be removed the same way. The results of the pilot study seem to demonstrate that only minimal peeling effects did occur on wet SLA titanium surfaces. Moreover, it seems like the biofilms were pushed even further into the deepest lacunae of the SLA discs. This could potentially be explained by the hydrophobic characteristic of the VPS. Knowing that polyether is hydrophilic, when compared to VPS, it is possible that better mechanical peeling effects would have been achieved with such material.

Chapter 7: Conclusion & Future Directions

7.1 Conclusion

Based on the results of this study, this mature multispecies biofilm model mimics well the in vivo biofilms and may be useful for the evaluation of the decontamination / disinfection of SLA implant surface. Most of the mature biofilm were easily removed, leaving only a very small proportion of bacteria on the SLA titanium surface after the first set of saline rinse. The tested chemical agents (CHX, UltraEtch, Tetracycline paste, Cetrimide-CHX-EDTA) did not improve the decontamination effect when compared with the 0.9% NaCl rinse, nor did the peeling effect of the VPS. A slight beneficial killing effect of CHX 1% gel or UltraEtch 35% might improve the disinfection of SLA implant surface, but the disinfection effect of Cetrimide-CHX-EDTA was not tested. Common implant decontamination protocols appear to be ineffective and better solutions for implant decontamination are crucially needed. Although, only SLA titanium discs were used in this study, there is no reason to believe the results would have been different on any other titanium surfaces.

7.2 Future Direction

Future studies should try to determine the effect of the rinse flow rate on the removal of the bacteria from the SLA implant surface contaminated with mature multi-species biofilms, or if the mechanical rinsing effect is also limited and reaching a plateau.

Chemotherapeutics agents with different wettability capacity and different viscosity should also be tested using this model to measure the impact of the different characteristics on the decontamination effect (biofilms removal). The impact of time on surface wettability should

also be assessed in order to find out if better decontamination or disinfection is achieved with longer treatment time (longer contact between biofilms and chemotherapeutic agents). Mechanical peeling with a more hydrophilic impression material should also be tested using this biofilms models.

Other therapy frequently reported in the literature in the treatment of peri-implantitis, such as abrasive air, laser, photodynamic therapy and plasma jet should also be tested using the proposed model. If none of these proposed therapies provide a better decontamination effect when compare to NaCl rinse, future studies should use the CLSM to assess the disinfection effect of chemical agents.

It would also be interesting to measure the capability of the remaining bacteria to repopulate the titanium disc after decontamination or disinfection protocol. It would be even more interesting to proceed with bioassay using gingival stem cells. Such experiment would reflects healing response after flap closure during peri-implantitis surgery in which stem cells will contact chemically cleaned / disinfected SLA titanium surface. The ideal chemical disinfectant shall remove enough biofilms, if not completely, in order to slowdown its repopulation and allow biocompatible surface for human cell attachment.

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Appendix

Appendix A : Statistical Analysis

A.1 Main Study

Dependent Variable		(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
Count	Tukey HSD	Rinse	Double Rinse	176.778*	61.323	.047	1.63	351.92
			CHX	155.889	61.323	.101	-19.25	331.03
			ETCH	122.333	61.323	.287	-52.81	297.48
			TETRA	159.222	61.323	.090	-15.92	334.37
		Double Rinse	Rinse	-176.778*	61.323	.047	-351.92	-1.63
			CHX	-20.889	61.323	.997	-196.03	154.25
			ETCH	-54.444	61.323	.900	-229.59	120.70
			TETRA	-17.556	61.323	.998	-192.70	157.59
		CHX	Rinse	-155.889	61.323	.101	-331.03	19.25
			Double Rinse	20.889	61.323	.997	-154.25	196.03
			ETCH	-33.556	61.323	.982	-208.70	141.59
			TETRA	3.333	61.323	1.000	-171.81	178.48
		ETCH	Rinse	-122.333	61.323	.287	-297.48	52.81
			Double Rinse	54.444	61.323	.900	-120.70	229.59
			CHX	33.556	61.323	.982	-141.59	208.70
			TETRA	36.889	61.323	.974	-138.25	212.03
		TETRA	Rinse	-159.222	61.323	.090	-334.37	15.92
			Double Rinse	17.556	61.323	.998	-157.59	192.70
			CHX	-3.333	61.323	1.000	-178.48	171.81
			ETCH	-36.889	61.323	.974	-212.03	138.25

Dependent Variable		(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
Percentage	Tukey HSD	Rinse	Double Rinse	37.1735*	10.5938	.009	6.91656	67.4305
			CHX	32.7562*	10.5938	.028	2.49920	63.0132
			ETCH	24.6262	10.5938	.158	-5.6307	54.8832
			TETRA	32.9896*	10.5938	.027	2.73267	63.2467
		Double Rinse	Rinse	-37.173*	10.5938	.009	-67.430	-6.91656
			CHX	-4.4173	10.5938	.993	-34.674	25.8396
			ETCH	-12.5473	10.5938	.760	-42.804	17.7097
			TETRA	-4.18389	10.5938	.995	-34.440	26.0731
		CHX	Rinse	-32.756*	10.5938	.028	-63.013	-2.49920
			Double Rinse	4.41736	10.5938	.993	-25.839	34.6743
			ETCH	-8.12994	10.5938	.938	-38.386	22.1270
			TETRA	.233469	10.5938	1.000	-30.023	30.4904
		ETCH	Rinse	-24.626	10.5938	.158	-54.883	5.63075
			Double Rinse	12.5473	10.5938	.760	-17.709	42.804
			CHX	8.12994	10.5938	.938	-22.127	38.3869
			TETRA	8.36341	10.5938	.932	-21.893	38.6204
		TETRA	Rinse	-32.989*	10.5938	.027	-63.246	-2.73267
			Double Rinse	4.18389	10.5938	.995	-26.073	34.4409
			CHX	-.233469	10.5938	1.000	-30.490	30.0235
			ETCH	-8.36341	10.5938	.932	-38.620	21.8936

*. The mean difference is significant at the 0.05 level.

A.2 CLSM Pilot Study

Dependent Variable: Percentage	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tamhane	Control	Rinse	-.0010	.13000	1.000	-.4806	.4787
		Double Rinse	.2121	.26961	1.000	-.7765	1.2007
		CHX	11.3575*	2.37742	.020	1.6161	21.0990
		ETCH	6.4352*	1.28548	.015	1.1913	11.6791
		TETRA	3.4953	.99982	.111	-.5669	7.5575
	Rinse	Control	.0010	.13000	1.000	-.4787	.4806
		Double Rinse	.2131	.24856	1.000	-.7727	1.1989
		CHX	11.3585*	2.37512	.021	1.6123	21.1047
		ETCH	6.4362*	1.28123	.015	1.1839	11.6885
		TETRA	3.4963	.99435	.111	-.5763	7.5689
	Double Rinse	Control	-.2121	.26961	1.000	-1.2007	.7765
		Rinse	-.2131	.24856	1.000	-1.1989	.7727
		CHX	11.1454*	2.38684	.023	1.4223	20.8686
		ETCH	6.2231*	1.30282	.017	1.0072	11.4390
		TETRA	3.2832	1.02201	.149	-.7493	7.3157
	CHX	Control	-11.3575*	2.37742	.020	-21.0990	-1.6161
		Rinse	-11.3585*	2.37512	.021	-21.1047	-1.6123
		Double Rinse	-11.1454*	2.38684	.023	-20.8686	-1.4223
		ETCH	-4.9223	2.69754	.767	-14.6768	4.8321
		TETRA	-7.8622	2.57370	.156	-17.4888	1.7643
	ETCH	Control	-6.4352*	1.28548	.015	-11.6791	-1.1913
		Rinse	-6.4362*	1.28123	.015	-11.6885	-1.1839
		Double Rinse	-6.2231*	1.30282	.017	-11.4390	-1.0072
		CHX	4.9223	2.69754	.767	-4.8321	14.6768
		TETRA	-2.9399	1.61996	.755	-8.5599	2.6801
	TETRA	Control	-3.4953	.99982	.111	-7.5575	.5669
		Rinse	-3.4963	.99435	.111	-7.5689	.5763
		Double Rinse	-3.2832	1.02201	.149	-7.3157	.7493
		CHX	7.8622	2.57370	.156	-1.7643	17.4888
		ETCH	2.9399	1.61996	.755	-2.6801	8.5599

Based on observed means.
The error term is Mean Square (Error) = 12.507.

*. The mean difference is significant at the 0.05 level.

A.3 SEM Pilot Study

	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Dependent Variable: Count							
Tukey HSD	Rinse	Double Rinse	67.000	90.183	.877	-221.80	355.80
		CCE	70.667	90.183	.860	-218.13	359.46
		Peeling	-16.333	90.183	.998	-305.13	272.46
	Double Rinse	Rinse	-67.000	90.183	.877	-355.80	221.80
		CCE	3.667	90.183	1.000	-285.13	292.46
		Peeling	-83.333	90.183	.793	-372.13	205.46
	CCE	Rinse	-70.667	90.183	.860	-359.46	218.13
		Double Rinse	-3.667	90.183	1.000	-292.46	285.13
		Peeling	-87.000	90.183	.772	-375.80	201.80
	Peeling	Rinse	16.333	90.183	.998	-272.46	305.13
		Double Rinse	83.333	90.183	.793	-205.46	372.13
		CCE	87.000	90.183	.772	-201.80	375.80
Dependent Variable: Percentage							
Tukey HSD	Rinse	Double Rinse	11.050027	14.873425	.877	-36.57991	58.67997
		CCE	11.654755	14.873425	.860	-35.97519	59.28470
		Peeling	-2.693788	14.873425	.998	-50.32373	44.93615
	Double Rinse	Rinse	-11.050027	14.873425	.877	-58.67997	36.57991
		CCE	.604728	14.873425	1.000	-47.02521	48.23467
		Peeling	-13.743815	14.873425	.793	-61.37376	33.88613
	CCE	Rinse	-11.654755	14.873425	.860	-59.28470	35.97519
		Double Rinse	-.604728	14.873425	1.000	-48.23467	47.02521
		Peeling	-14.348543	14.873425	.772	-61.97848	33.28140
	Peeling	Rinse	2.693788	14.873425	.998	-44.93615	50.32373
		Double Rinse	13.743815	14.873425	.793	-33.88613	61.37376
		CCE	14.348543	14.873425	.772	-33.28140	61.97848