Peri-Implantis: Associated Microbiota, Biofilm Formation and Decontamination

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PERI-IMPLANTITIS
ASSOCIATED MICROBIOTA, BIOFILM FORMATION AND DECONTAMINATION

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ABSTRACT

Peri-implantitis is a bacterially induced inflammatory condition that results in resorption of the bone supporting an implant. The methods for treating it are mainly empirical, and none have been shown to be universally successful.

The first part of this research aimed to identify and compare the composition of the microbiota around dental implants with and without peri-implantitis. Subgingival plaque samples were collected from twenty-one subjects and samples were cultured using non-selective media and bacterial identification was carried out using 16S rRNA PCR.

The second phase of the study investigated surface characteristics of implants that could influence the formation of a biofilm. Using a clinically relevant organism (Streptococcus oralis), an in vitro biofilm was developed and biofilm formation on four titanium implant surfaces exhibiting varying degrees of roughness with and without fluoride ions incorporated was evaluated using confocal laser scanning electron microscopy, bacterial culturing and scanning electron microscopy.

The final phase examined the effects of mechanical debridement and air-polishing using glycine or 45S5 bioactive glass powders in re-establishing biocompatibility of the previously contaminated implant surfaces. Biocompatibility of the surfaces was tested using cell culture with alveolar human osteoblasts for cell viability, proliferation and cell differentiation.

Results showed that a complex subgingival microbiota was found around dental implants. Peri-implantitis was associated with an increased bacterial load. Filifactor alocis, Streptococcus constellatus, Parvimonas micra and Actinomyces meyeri were significantly more prevalent in peri-implantitis, while Veillonella parvula/ dispar and Neisseria elongata were more prevalent in healthy sites.

There were no significant differences in biofilm volume, surface area covered by the biofilm, nor number of bacteria found on any of the surfaces tested or at different levels within the biofilm suggesting that once colonization has been established, the effect of the surface diminishes with maturation of the biofilm.

Although cleaning efficiency was different, all decontamination methods tested were shown to yield a biocompatible implant surface.
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LIST OF PRESENTATIONS

ORAL PRESENTATION

EUROPERIO 7 – VIENNA 6 – 9, JUNE 2012

The Microflora Around Failing And Healthy Dental Implants


POSTER PRESENTATION (ABSTRACT ACCEPTED)

EAO (EUROPEAN ASSOCIATION OF OSSEOINTEGRATION) ANNUAL SCIENTIFIC CONGRESS DUBLIN 17 – 19, OCTOBER 2013

In vitro biofilm formation on four different titanium implant surfaces
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADI</td>
<td>Association of Implantology, UK</td>
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<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>BAG</td>
<td>Bioactive glass</td>
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<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>BOP</td>
<td>Bleeding on probing</td>
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<tr>
<td>CBL</td>
<td>Crestal bone loss</td>
</tr>
<tr>
<td>CFUs</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal Laser scanning Microscopy</td>
</tr>
<tr>
<td>CP</td>
<td>Commercially pure</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DPCM</td>
<td>Dark Phase Contrast Microscopy</td>
</tr>
<tr>
<td>DPT</td>
<td>Dental Panoramic Topography</td>
</tr>
<tr>
<td>EDS</td>
<td>Energy Dispersive X-ray Spectroscopy</td>
</tr>
<tr>
<td>EWOP</td>
<td>European Workshop on Periodontology</td>
</tr>
<tr>
<td>FAA</td>
<td>Fastidious Anaerobic Agar</td>
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<tr>
<td>FAB</td>
<td>Fastidious Anaerobic Broth</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FISH</td>
<td>Fluorescent In situ Hybridization</td>
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<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HOB</td>
<td>Human osteoblasts</td>
</tr>
<tr>
<td>HOMD</td>
<td>Human Oral Microbiome Database</td>
</tr>
<tr>
<td>mFUM</td>
<td>modified Fluid Universal Medium</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>N.D.</td>
<td>not detected</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OH</td>
<td>Oral hygiene</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pdl</td>
<td>Periodontal</td>
</tr>
<tr>
<td>PID</td>
<td>Peri-implant diseases</td>
</tr>
<tr>
<td>PPD (PD)</td>
<td>Probing pocket depths</td>
</tr>
<tr>
<td>Ra</td>
<td>Arithmetical mean roughness (profile, 2D)</td>
</tr>
<tr>
<td>RDP</td>
<td>Ribosomal Database Project</td>
</tr>
<tr>
<td>Rtm</td>
<td>Mean roughness depth (mean peak to valley heights)</td>
</tr>
<tr>
<td>Sa</td>
<td>Average roughness over the whole area (3D)</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SFE</td>
<td>Surface free energy</td>
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<tr>
<td>SLA</td>
<td>Sand-blasted Acid-etched</td>
</tr>
<tr>
<td>Sm</td>
<td>Mean spacing between peaks</td>
</tr>
<tr>
<td>Ti</td>
<td>Titanium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------------------</td>
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<tr>
<td>TPS</td>
<td>Titanium plasma sprayed</td>
</tr>
<tr>
<td>UHQ</td>
<td>Ultra high quality</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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</table>
PREFACE

This thesis is based on the following three studies:

I. The microbiota associated with peri-implantitis and healthy dental implants.

II. *In vitro* biofilm formation on different implant surfaces.

III. Re-establishment of biocompatibility of previously contaminated implant surfaces.
CHAPTER 1  GENERAL INTRODUCTION
1.1 History of Dental Implants

People have attempted to replace missing teeth with suitable synthetic materials since the start of human civilizations and archaeological findings show that attempts using seashells, ox teeth and human teeth date back to as early as the ancient Egyptian civilization.

However, dental implants in their current form today were developed in 1952 by Dr. Per-Ingvar Brånemark, who discovered inadvertently that titanium chambers which he inserted into the tibia of rabbits to study the microcirculation during bone healing, could not be removed for further use and that a strong anchorage of the titanium to bone occurred. The application of a force of 100 kilograms was unable to dislodge the implants, and fracturing of the implant site was necessary (Sullivan, 2001).

Brånemark named this phenomenon osseointegration and defined it as a “direct structural and functional connection between ordered living bone and the surface of a load-bearing artificial implant”.

At about the same time, the Swiss scientist Andre Shroeder described the same phenomenon, which he called “functional ankylosis” to describe the rigid fixation of the implant to the jaw bone, and stated that new bone is laid down directly upon the implant surface, provided that the rules for atraumatic implant placement are followed and the implant exhibits primary stability (Schroeder and Belser, 1996, Schroeder et al., 1981).

Basic and animal research was conducted in the years that followed and 10-year implant integration with no adverse reactions was initially established in dogs. In 1965, Gösta Larsson, who presented with a secondary cleft and an edentulous mandible, became the first human patient in which dental implants were installed.

But other implant treatments that were carried out during the same period (subperiosteal and blade implants) (Linkow and Mahler, 1975, Small, 1986) were unpredictable and results were erratic. They accepted the existence of a connective tissue capsule surrounding the implant acting as a "pseudo-periodontium" and such implants anchored in fibrous tissue ended up with high clinical failure rates. The scientific community met Brånemark’s findings with disbelief and ridicule, and suggested that titanium should not be used in dogs, let alone in humans (Branemark, 2005, Shulman and Driskell, 1997). It was not until the Toronto Conference on Osseointegration in Clinical Dentistry in 1982...
that treatment using titanium dental implants became accepted. The conference introduced the concept of osseointegration along with its applications. This was supported by the work published by Brånemark (Adell et al., 1981) which described the placement of 2768 implants in 410 jaws of 371 patients during the 15 year period from 1965-1980. A group of these patients (130 jaws, 895 implants) were followed for 5 – 9 years and 81% of the maxillary and 91% of the mandibular implants remained stable, supporting fixed bridges.

This was a turning point for implant dentistry and brought it from a shunned field into one of dentistry’s most significant and important developments. It succeeded in changing traditional beliefs and promoted a profound academic and research interest, which has revolutionized dental care (McClarence, 2003).

Consequently, in 1986, Albrektsson proposed the following criteria for implant success which have been the benchmark for many years (Albrektsson et al., 1986):

1) The individual unattached implant is immobile when tested clinically

2) No evidence of peri-implant radiolucency is present as assessed on an undistorted radiograph

3) Mean vertical bone loss is less than 0.2 mm annually after the 1st year of function or service.

4) There is no persistent pain, discomfort or infection attributable to the implant

5) Implant design does not preclude placement of a crown or prosthesis with an appearance that is satisfactory to the patient or dentist

6) There is an 85% success rate at the end of a five year post restorative period, with an 80% success rate at the end of 10 years post restorative function

Dental implants have now come a long way since they were first introduced to the dental profession. In many cases, they can be considered the preferred treatment option for the replacement of missing teeth and provide a predictable treatment with average reported success rates of 95% (Albrektsson, 1988, Ellegaard et al., 1997, Vajdovich and Fazekas, 1999, Zarb and Zarb, 2002).
However failure sometimes occurs and when that happens, it is traumatic, expensive, involves prolonged treatment time and is frustrating and psychologically depressing to both patient and dentist (Berglundh et al., 2002).

Failures in implant dentistry can be chronologically divided into early and late failures (Esposito et al., 1998). Early failures have been attributed to surgical trauma, inadequate bone volume (Jaffin and Berman, 1991), lack of primary stability, intra-osseous infection and bacterial contamination of the recipient site, while late failures have been attributed to peri-implantitis and/or biomechanical overload with substantial evidence supporting the former as the primary factor (Lang et al., 2011).
1.2 PERI-IMPLANT DISEASES

1.2.1 DEFINITION AND PREVALENCE OF PERI-IMPLANT DISEASES

The sixth European Workshop on Periodontology EWOP (2008) has confirmed that peri-implant diseases are infectious in nature. Peri-implant mucositis describes an inflammatory lesion that resides in the mucosa, while peri-implantitis also affects the supporting bone (Lindhe et al., 2008).

While these definitions are adequate, the diagnostic clinical criteria for them are less clear. It has been identified that the key parameter for the diagnosis of peri-implant mucositis is bleeding on gentle probing (< 0.25 N). Peri-implantitis however is characterized by changes in the crestal bone level in conjunction with bleeding on probing with or without concomitant deepening of peri-implant pockets and suppuration (Lang et al., 2011). Suppuration has been encountered more frequently in implants with “progressive” bone loss, particularly in smokers, and may be associated with episodes of active tissue destruction (Speechley, 2012).

A wide variation in the prevalence of peri-implantitis has been reported in different studies. This has ranged between 28% - 56% of subjects and 12% - 43% of implants (Fransson et al., 2005, Fransson et al., 2008, Roos-Jansaker et al., 2006b, Zitzmann, 2008 #569). A recent review reports that the prevalence of peri-implantitis occurs in 10% of implants and 20% of patients, 5 – 10 years following implant placement (Mombelli et al., 2012). These variations, in part could be due to the historical misconception that probing around implants should be avoided (Ericsson and Lindhe, 1993) leading to peri-implantitis being under-diagnosed. More importantly, these variations can be due to the lack of a consistent definition for the disease and the differences in disease criteria since authors have used different definitions of peri-implantitis in clinical studies. For example, Behneke et al. (Behneke et al., 2002) suggested that peri-implantitis should include presence of clinical inflammation (bleeding, redness, swelling and pus) and progressive bone loss (not defined in millimetres). Ekelund et al. (Ekelund et al., 2003) described peri-implantitis as a combination of inflammation, pain and continuous bone loss. Ferreira et al. (Ferreira et al., 2006) suggested that the diagnosis of peri-implantitis had to meet the following criteria: (i) PPD ≥ 5mm, (ii) BOP and (iii) vertical bone loss. Karoussis et al. (Karoussis et al., 2004) presented almost similar criteria namely, PPD ≥ 5 mm and BOP (or pus) and radiographic bone loss. Roos-Jansaker et al. (Roos-Jansaker et al., 2006b), Laine et
al. (Laine et al., 2006) and Renvert et al. (Renvert et al., 2007) suggested that peri-
implantitis required bone loss more than 1.8 mm after the first year of function in
combination with bleeding and/ or suppuration.

Furthermore, an additional confounding factor in the diagnosis of peri-
implantitis is the time point used to establish baseline records. It is now agreed that peri-implant probing
assessments and a radiograph should be obtained at the time of prosthesis installation to
account for physiologic bone remodelling that occurs after implant placement.
Therefore when changes in the clinical parameters indicate disease (BOP, increased
PPD), further radiographs are recommended to evaluate possible bone loss (Lindhe et
al., 2008).

Last but not least, the most commonly used outcome measures to assess the
predictability and validity in implant dentistry are survival and success of implants.
Survival statistics use the “presence or absence” of implants as end-point outcome
measures, and hence do not take into consideration the presence of biological problems
arising from peri-implant diseases. This, in addition to the lack of universally accepted
and standardized success criteria makes the comparison of data between studies difficult
and this limits the usefulness of systematic reviews (Needleman et al., 2012, Sennerby
and Becker, 2000).

The limitations in the reporting of implant success have been summarized by Tonetti
and Palmer (Tonetti et al., 2012) and can be attributed to:

• Lack of consensus in the definition of success for implant therapy
• Lack of consensus in the choice of outcomes to measure implant success
• Validity and relevance of the outcome measures (e.g. difficulty in measuring
probing depths due to implant designs (platform switching) and/ or restorative
contours that may hinder effective probing)
• Lack of clarity of characteristics of study or reference groups.
• Variation in the timing of outcome assessment (e.g. timing of baseline
observation)
• Lack of details in reporting (the extent as well as the severity i.e. amount of bone loss) of the lesion (Lang et al., 2011).

• When peri-implantitis is reported, the implant is usually used as the unit of analysis although it has been recognized that the outcomes of individual implants, like teeth are usually clustered within individuals (Klinge et al., 2005). Thus it is important to also report prevalence on a ‘per subject’ or ‘per patient’ basis. This in turn minimizes type 1 error which could occur when the implant is used as the unit of analysis, since increasing the “n” number of implants would increase the precision of the study and may create spurious statistically significant findings (Needleman et al., 2012)

It is obvious that establishing clear success criteria to define implant performance is important. Accordingly, Needleman et al. (Needleman et al., 2012) have indicated that these criteria should be summarized according to the definition by Albrektsson et al. (Albrektsson et al., 1986) and adapted by Buser et al. (Buser et al., 1990) as well as Karoussis et al. (Karoussis et al., 2004), Roos- Jansaker et al. (Roos-Jansaker et al., 2006a, Roos-Jansaker et al., 2006c) and Renvert et al. (Renvert et al., 2007) as follows:

• Absence of mobility (Buser et al., 1990).

• Absence of persistent subjective complaints (pain, foreign body sensation and/or dysaesthesia) (Buser et al., 1990).

• Absence of recurrent peri-implant infection with suppuration (Buser et al., 1990).

• Absence of a continuous radiolucency around the implant (Buser et al., 1990).

• No PPD > 5 mm (Bragger et al., 2001, Mombelli and Lang, 1994). 

• No PPD > 5 mm and bleeding on probing (BOP) (Mombelli and Lang, 1994)

• After the first year of service, the annual vertical bone loss should not exceed 0.2 mm (mesially or distally) (Albrektsson et al., 1986, Albrektsson and Isidor, 1994).

• Progressive bone loss plus bleeding on probing.
Based on this, the Association of Implantology, UK (ADI)’s expert panel consensus meeting, held in 2012 in London, suggested the following classification for implant success or failure (Table 1).

<table>
<thead>
<tr>
<th>Success</th>
<th>Failing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stable marginal bone levels after an initial crestal bone loss of &lt; 1.5mm</td>
<td>&lt; 50% progressive bone loss which has not stabilized</td>
</tr>
<tr>
<td>No BOP</td>
<td>Deep pocketing</td>
</tr>
<tr>
<td>PD &lt; 4mm</td>
<td>BOP</td>
</tr>
<tr>
<td>Good plaque control</td>
<td>Less than ideal plaque control</td>
</tr>
<tr>
<td>Inflamed peri-implant mucosa</td>
<td>Healthy peri-implant mucosa</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>At Risk</th>
<th>Failed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial bone loss &lt; 4mm but bone loss has stabilized</td>
<td>Deep pocketing</td>
</tr>
<tr>
<td>PD &lt; 5mm</td>
<td>Discharge</td>
</tr>
<tr>
<td>No BOP immediate or delayed</td>
<td>BOP</td>
</tr>
<tr>
<td>Plaque index less than ideal</td>
<td>Mobility</td>
</tr>
</tbody>
</table>

**Table 1 Implant success and failure definition according to ADI.**

Failing implants often demonstrate few if any patient perceptible signs or symptoms. It is thus the responsibility of the clinician to recognise the disease especially in it’s early and reversible stages. When clinical signs are overlooked, implant mobility may be the first symptom perceived by the patient, by which time all treatment options are ineffective and the implant has failed (Speechley, 2012).
1.2.2 Similarities and differences between periodontitis and peri-implantitis lesions

Since both diseases represent a host response to a bacterial challenge, there is no reason to assume that the response will be different in the peri-implant mucosa when compared to the gingiva (Lang et al., 2011). Most of our knowledge comparing similarities and differences of peri-implant and periodontal lesions stems from animal studies using plaque-retaining ligatures. Berglundh, Zitzmann and Donati (Berglundh et al., 2011) indicate that microbiologically and clinically, both lesions have considerable resemblance, where initial biofilm formation led to lesions similar in size and composition after 3 weeks. However histopathologically, the apical extension of the lesions in peri-implantitis were more pronounced, extending consistently apical of the pocket epithelium and was in direct contact with the biofilm residing on the implant surface. On the contrary, a “self-limiting” process existed in the tissues around teeth resulting in a protective connective tissue capsule separating the lesion from the alveolar bone. A similar connective tissue capsule could not be found in biopsies from the peri-implant tissues and the lesion extended to the bony crest. Plasma cells and lymphocytes dominated both types of lesions, but neutrophil, granulocytes and macrophages occurred in larger proportions in peri-implantitis. Also peri-implantitis, in contrast to periodontitis lesions, exhibited large amounts of osteoclasts lining the surface of the crestal bone.

A clinically noticeable feature is that submucosal calculus formation is less common on implants than on teeth and that peri-implantitis in general progresses faster than periodontitis. One factor that could explain the difference in progression of inflammation is the anatomical difference of the gingival architecture around implants and natural teeth. Histologically, around implants the circular supracrestal collagen fibres are parallel to the implant surface. In contrast, collagen fibres around teeth have insertions in cementum, which may provide greater protection against the spread of inflammation (Berglundh et al., 1991).

Another difference is the circumferential nature of the intrabony defects commonly observed in peri-implantitis compared to the localized periodontitis lesions. Bone loss around implants often occurs as a saucer-shaped defect, which can be observed radiographically. The reason for this is unknown, but it may be speculated that the absence of a periodontal ligament and the lack of anchorage of collagen fibres may lead
to lateral spread of infection, determining the morphology of the lesion and explaining the characteristic bony defects seen around implants (Berglundh et al., 1991).

1.2.3 Risk factors for peri-implant diseases

The systematic review by Heitz-Mayfield summarizes the available evidence for risk factors associated with peri-implant diseases (Heitz-Mayfield, 2008). She concluded that poor oral hygiene (Ferreira et al., 2006, Lindquist et al., 1996), a history of periodontitis (Van der Weijden et al., 2005) and cigarette smoking (Strietzel et al., 2007) are major risk factors for the development of peri-implant disease. Limited evidence existed linking peri-implant disease to alcohol consumption and diabetes. There was also limited and conflicting evidence associating genetic traits and implant surfaces with the development of peri-implant diseases. Nevertheless all the above may co-exist and their effects may be synergistic (Table 2)

<table>
<thead>
<tr>
<th>Risk Indicator</th>
<th>Peri-implantitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of periodontitis</td>
<td>+</td>
</tr>
<tr>
<td>Poor oral hygiene</td>
<td>+</td>
</tr>
<tr>
<td>Microorganisms</td>
<td>+</td>
</tr>
<tr>
<td>Smoking</td>
<td>+</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>(+)</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>(+)</td>
</tr>
<tr>
<td>Implant design</td>
<td>(+)</td>
</tr>
<tr>
<td>Implant surface</td>
<td>(+)</td>
</tr>
<tr>
<td>Residual cement</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2 Risk Indicators for peri-implantitis; + strong evidence, (+) limited evidence
1.2.4 The Microbiota around Dental Implants

It appears that the main contributory factor for peri-implantitis, as in periodontitis, is the accumulation of bacterial dental plaque (Schou et al., 1992, Lang et al., 2011). Bacteria in plaque have the ability to initiate an inflammatory response that may lead to bone destruction. Whether this progresses to involve the supporting tissues (whether around teeth or implants) seems to be related to an interaction of factors e.g. microbiological, genetic susceptibility and immune response (Socransky and Haffajee, 1992).

Due to the important role of the plaque biofilm in the initiation and progression of peri-implantitis (Lang et al., 2011), the microbiology of the peri-implant tissues has attracted much attention in the scientific community. Although numerous studies have been published, research in this area has produced conflicting results and which species are responsible for peri-implantitis remains unclear.

As mentioned previously, the definition of peri-implantitis was introduced at the first EWOP in 1993 (Lang and Karring, 1993). Later, at the sixth EWOP in 2008 (Lindhe et al., 2008), the distinction was made between peri-implantitis and peri-implant mucositis, both being peri-implant diseases. Studies before those dates used various terms to describe such implants, for example unsuccessful, diseased, failing, etc. Peri-implantitis is now considered a more accurate term, that best describes the disease. In this review, the terms that were used in their original context will be referred to, as inclusion criteria in the early studies were not always clear.

Similarly, many bacterial species have been renamed and reclassified. In the following review, the names used in the original text will be referred to.

To determine the impact of microorganisms on peri-implant pathology, data from different kinds of studies need to be evaluated. Association studies can reveal which bacteria are detected more frequently and at higher levels in diseased sites than in health. Longitudinal investigations are required for the study of early colonization of implants, to determine the range of normal fluctuation in bacterial populations and to possibly demonstrate microbial changes prior to or concomitant with disease development. Treatment studies may answer the question of whether reducing or eradicating suspected organisms would improve the clinical conditions of diseased tissues.
Early studies mainly used dark field microscopy, phase contrast microscopy, differential phase microscopy, and selective and non-selective culture media to identify organisms.

Lekholm et al. (Lekholm et al., 1986b) carried out one of the earliest longitudinal studies regarding the microbiota around healthy dental implants. They reported on the microbial composition associated with combined prostheses i.e. those that have natural teeth abutments connected to implants. Supra and subgingival plaque samples were taken from 10 partially edentulous patients who had fixed bridge restorations that had been in function for an average of 18 months. Bright – light phase contrast microscopic examination showed primarily cocci and non-motile rods with an absence of spirochaetes. The microbiota was similar for both tooth and titanium abutments. Cultural analysis revealed predominantly 80% of the Gram - positive facultative cocci were streptococcal isolates, the most predominant species being *Streptococcus sanguinis*, *Streptococcus mitis* and *Streptococcus acidominimus*. The remaining 20% of the Gram-positive facultative cocci were catalase-positive and belonged to the *Staphylococcus* and *Micrococcus* group of organisms. The Gram-positive anaerobic cocci were members of *Peptococcus* and *Peptostreptococcus* species. Concerning the Gram-positive facultative rods, about 50% of the isolates were catalase-positive organisms which were tentatively identified as *Actinomyces viscosus* and the other half were catalase-negative organisms, which were presumed to be *Actinomyces naeslundii*. All Gram-negative anaerobic cocci were considered *Veillonella parvula*. Among the Gram-negative anaerobic rods, *Fusobacterium nucleatum* was the most predominant species.

The same authors conducted a retrospective study (Lekholm et al., 1986a) on 20 edentulous patients who were selected at random from a pool of 400 subjects treated during the period between 1965 to 1981. The patients had a total of 125 implants supporting fixed bridges that had been in function for an average of 7.6 years. 40% of probing depths were 3 mm or less, 45% were between 4 and 5 mm and 15% were more than 6 mm. Samples of the subgingival microbiota were obtained using curettes from sites having the deepest and shallowest pockets in each jaw. Coccoid cells and non-motile rods dominated the microbiota with a total frequency of 94%, while spirochaetes and motile rods were present in 1.5% only. Deeper pockets were correlated with increasing presence of spirochaetes.
Using the same Brånemark implant system, Bower et al. (Bower et al., 1989) examined 10 supragingival and 9 subgingival plaque samples from around healthy implants, that had been in function for 3 years supporting fixed mandibular prostheses. The samples were collected using scalers and were examined under phase contrast microscopy. Results showed that coccoid and non-motile rods predominated all samples (supragingival 89% and subgingival 85%) while spirochaetes and motile rods were not found in the supragingival samples and was found in only one of the subgingival samples.

Mombelli et al. (Mombelli et al., 1987) presented some of the earliest data associated with unsuccessful implants. They compared the subgingival microbiota surrounding healthy and failing plasma-coated titanium hollow cylinder implants in edentulous patients that had been wearing overdentures with bar or ball attachments, for over one year. They divided the subjects into 2 groups; a successful implants group, comprising 5 patients each with 2 healthy implants and a failing implants group of 7 patients, each with at least one failing implant and one healthy control implant. Failing implant sites were characterized by pocket formation > 6mm, suppuration and radiographic evidence of bone loss. Using dark field microscopy and culture, they found that failing implant sites had a significantly higher proportion of the micro-organisms traditionally associated with periodontal diseases. These were Gram-negative anaerobic rods, including *Prevotella* spp., *Fusobacterium* spp., spirochaetes and *Wolinella* spp. This was noticeably different from subgingival plaque from the clinically healthy dental implants, which were similar regardless of which patient group they came from and consisted predominantly of cocci. On the basis of these findings, peri-implantitis has been suggested to be a “site-specific”.

The following year, Mombelli et al. (Mombelli et al., 1988), published another study that looked at bacterial colonization over a 180 day period of 9 newly inserted titanium hollow screw implants in 5 totally edentulous patients. The day before implant placement, microbial samples were obtained from the mucosal surface, thereafter samples were taken from the peri-implant pockets using paper points at weekly intervals for 8 weeks. Additional samples were obtained at 90, 120, 150 and 180 days after implant placement and were analyzed using dark field microscopy and culture. After insertion of the implants, gram-positive coccoid cells accounted for over 80% of the total organisms. This high level of Gram-positive bacteria was maintained throughout
the study at all sites except one. Predominant isolates included *A. naeslundii*, *Veillonella* sp. and *Actinomyces odontolyticus*. One implant demonstrated clinical breakdown with increased probing depth, bleeding and suppuration. The failing implant showed a decrease in coccoid cells and an increase in motile rods after 21 days. *A. odontolyticus* was first detected after 21 days, *Fusobacterium* spp. were found for the first time on day 42 and finally spirochaetes were identified in the sample taken at day 120, when pus formation was clinically evident. Black pigmented anaerobes were infrequently found.

In a follow up study, Mombelli and Mericske-Stern (Mombelli and Mericske-Stern, 1990) studied the microbiota from 18 edentulous patients, again with overdentures and with the same type of implants, 30 months after placement. They found 52.8% were facultative anaerobic cocci and 17.4% facultative anaerobic rods, but only 7.3% were Gram-negative rods. *Fusobacterium* spp and *Prevotella intermedia* were both found in only 9% of the samples. *Porphyromonas gingivalis* and spirochaetes were not detected, suggesting that they were rarely isolated from edentulous individuals. Moreover, 9 of the patients were followed up to see if there were any microbiological changes during the third, fourth and fifth years, but no significant time trends could be identified.

Maxson et al. (Maxson et al., 1992) evaluated the microbiota associated with clinically stable implants by obtaining plaque samples from 14 completely edentulous patients that had been rehabilitated by either transosteal gold alloy implants (TMI system) or titanium endosseous implants (Brånemark). Cultures for selected organisms revealed *Actinomyces* to be present in high proportions whilst motile organisms and black-pigmented anaerobic rods were virtually undetectable.

In a widely cited study by Apse et al. (Apse et al., 1989), tooth and implant sites were compared in 15 partially edentulous patients (28 peri-implant and 19 periodontal sites) and 6 edentulous patients (13 implants). All implants and teeth assessed were clinically healthy and had been in function for an average of 16 and 44 months for the partially edentulous and edentulous groups respectively. The microbiological analysis included dark field microscopy, anaerobic culturing for total colony forming unit counts and identification of black-pigmented bacteria. Coccoid cells dominated all sites and non-motile rods, motile rods and spirochaetes occurred in very low numbers. The proportion of motile rods from implant sites was significantly higher in the edentulous than in the partially edentulous group. On the other hand, black-pigmented bacteria (p < 0.005) and
Capnocytophaga spp. (p < 0.001) occurred more frequently at implant sites in the partially edentulous group when compared to the edentulous implant sites. This was attributed to the possibility that there were lower numbers of disease-associated organisms in edentulous mouths. Considering these distinct differences, in addition to the notable similarities between the implant and periodontal sites in the same patient, it was suggested pockets around teeth might serve as a reservoir of microorganisms for the colonization of the subgingival environment around implants in partially edentulous patients. This was contrary to the idea that peri-implantitis was a site-specific infection as Mombelli et al. had proposed regardless of the patient (Mombelli et al., 1987).

The importance of staphylococci as one of the aetiological species was suggested by Rams et al. (Rams et al., 1990). They reported that a higher proportion of staphylococci (15.1%) were present in peri-implantitis lesions compared to gingivitis (0.06%) or periodontitis lesions (1.2%) potentially implicating these infrequently observed microorganisms. Samples from 13 patients with 20 titanium implants that demonstrated progressive pocketing, bone loss, and abscess formation, were obtained by inserting paper points for 10 seconds in the pockets. The bacterial samples were then plated onto Staphylococcus 110 medium and non-selective enriched Brucella blood agar and incubated anaerobically for 4 and 10 days respectively. Staphylococcal identification was based on colonial morphology, Gram-stain characteristics and a positive catalase reaction. Staphylococci were identified in 9 out of the 13 patients and 11 out of the 20 implants. One third of the staphylococcus species were identified as Staphylococcus aureus, while two thirds were identified as Staphylococcus epidermis using biochemical methods.

With the introduction of hydroxyapatite coatings to dental implants in the early nineties, it was inevitable that this would be accompanied by an interest in the surrounding microbial profile of these promising osteoinductive surfaces. Nakou and colleagues (Nakou et al., 1987) described the microbial colonization of 10 clinically healthy dental implants with a hydroxyapatite surface in 5 edentulous patients. The samples were taken from the supra and subgingival sites, two to ten weeks after implant placement from the deepest peri-implant pocket of each implant using a curette. The samples were investigated using non-selective and selective anaerobic culture media and dark field microscopy. The supragingival plaque was dominated by Gram-positive facultative cocci (streptococci) and rods and the sub-gingival plaque by Haemophilus spp. and V.
parvula. No black-pigmented *Bacteroides* were found in any of the examined samples. This was explained by the short time that the implants were present in the edentulous mouth and the shallow, inflammation-free, clinically healthy peri-implant crevices that might not provide the nutritional factors necessary for growth of these fastidious microorganisms.

Quirynen and Listgarten (Quirynen and Listgarten, 1990) analysed the distribution of bacterial morphotypes around natural teeth and healthy dental implants in 24 partially edentulous patients using differential phase contrast microscopy. They selected teeth and implants with comparable clinical indices and they found no significant microbiological differences between them. Ccocoid cells dominated all the implant and dental surfaces (65.8% and 56.5% respectively) and motile rods and spirochetes occurred in very low proportions. When fully edentulous patients were considered, significantly less motile rods and spirochetes were found when compared with implants in dentate patients. This may reflect the diverse ecological characteristics of the edentulous and the partially edentulous mouths. It may also indicate that the presence of teeth influences the composition of subgingival plaque around these implants, by serving as a source for the initial colonization of the implants. In the same study, the authors took 4 samples from failing implants. In the first 3, occlusal overload was deemed the cause of failure and samples showed a plaque containing no spirochaetes and few (0-3%) motile rods. However the fourth sample, which was accompanied by signs of inflammation, contained 11% spirochaetes and 16% motile rods.

Rosenberg et al. (Rosenberg et al., 1991) argued that there were 2 distinct types of implant failures, infectious and traumatic failures, each with a different entity of surrounding microorganisms. In this respect, infectious failures were implants with deep pockets and bone loss demonstrating clinical signs of infection (bleeding, suppuration and granulomatous tissue upon removal). Dark field microscopic observations of these samples exhibited a microbiota characteristic of a pathogenic milieu, in which spirochetes and motile rods made up 42% of the total morphotypes (21% each). The results of cultural analysis showed high proportions of *Peptostreptococcus micros*, enteric Gram-negative rods, *Fusobacterium* spp. and yeasts. Moderate levels of the *P. gingivalis*, *P. intermedia*, *Wolinella recta* and *Pseudomonas aeruginosa* were seen as well *Aggregatibacter actinomycetemcomitans*, *S. aureus* and *S. epidermis* were recovered in low proportions. On the other hand, failing implants that lacked the clinical
signs of infection demonstrated few or no major suspected periodontal pathogens and were termed traumatic failures.

Dharmar et al. (Dharmar et al., 1994) also compared the subgingival bacterial population around 64 healthy implants and 22 teeth in 18 partially edentulous and 6 totally edentulous patients. They used phase contrast microscopy to observe the morphological bacterial composition. Results showed a similar microbiota for teeth and dental implants in partially edentulous patients. Spirochaetes, although very few, were positively correlated with increasing pocket depths and were absent in totally edentulous patients, again suggesting the possibility of colonization of the implants from the bacterial reservoir of teeth in the same mouth.

Leonhardt et al. (Leonhardt et al., 1993) proposed a paradigm shift arguing that so-called periodontopathogens, such as \textit{P. gingivalis}, \textit{P. intermedia}, \textit{Capnocytophaga} spp., \textit{Campylobacter rectus}, and \textit{A. actinomycetemcomitans}, were also present in implants without any signs of destructive processes and could not be used as a marker, relating only to individuals with persisting deep pockets. In their study, 19 patients were provided with 63 implants and were sampled at baseline, 3, 6, 12, 24 and 36 months. Only 3 implants failed in one patient and this was accompanied by the presence of \textit{P. intermedia}. Selective media and phase-contrast microscopy were used to identify the organisms leading to the conclusion that these organisms were also found around healthy implants and that the initiation of peri-implantitis is a multi-factorial process that involves technical, anatomical, microbiological and host-related factors. These organisms may still be important in maintaining the destructive process around the implants, however their presence will not necessarily result in failure.

1.2.4.2 Molecular biology studies

With the advancement of microbiological identification techniques, more researchers started using DNA – DNA checkerboard hybridization and PCR techniques for the identification of the microbiota around implants.

Papaioannou and co-workers (Papaioannou et al., 1996), tested the hypothesis that teeth may act as a reservoir of microorganism using DNA probe analysis to test for the presence of 8 organisms, with disease associated potential, namely \textit{P. gingivalis}, \textit{P. intermedia}, \textit{Eikenella corrodens}, \textit{F. nucleatum}, \textit{Treponema denticola}, \textit{C. rectus}, \textit{Tannerella forsythia} and \textit{A. actinomycetemcomitans}. 

The study included 6 patients divided into 2 groups, 3 patients with chronic adult periodontitis and 3 with refractory periodontitis. In each patient, plaque samples were obtained using paper points from deep (>4.5mm) and shallow (<3.5mm) pockets from around implants and teeth. Results suggested that ‘infected’ pockets around teeth were responsible for the transmission of suspected organisms to pockets around implants. They showed differences in the subgingival flora between the chronic adult periodontitis as well as refractory periodontitis, as well as between deep and shallow pockets, around both implants and teeth within the same patient, where deep pockets harboured more spirochaetes and motile organisms than the respective shallow pockets. DNA probe analysis detected all organisms except for *A. actinomycetemcomitans* contrary to what had been suggested (Becker et al. 1990 and Rosenberg et al. 1991). They concluded that pockets around teeth with periodontal breakdown contained ‘pathogenic’ organisms that would lead to the colonization of implants placed. Although results were interesting and have been widely cited in other papers, the sample size was small (six patients out of which only 4 implants and 6 teeth with deep pockets were sampled) and only certain species were targeted and no healthy controls were included.

Quirynen et al. (2006) followed the dynamics of initial subgingival colonization of ‘pristine’ peri-implant pockets from 1 week till 78 weeks after abutment connection. The samples were analyzed by DNA-DNA hybridization, real-time PCR and/or culturing. In the 29 patients who completed the study, they observed that bacteria associated with periodontitis were found in healthy pockets within one week after abutment connection and that they increased only slightly after the second week and reached a more or less stable profile from 3 months onwards. They also found that deeper pockets, although not related to disease, harboured more suspected disease-associated species.

Shibli and colleagues (Shibli et al., 2008), compared the composition of the microbiota present in both supra and subgingival plaque in 44 subjects with and without peri-implantitis. They used checkerboard DNA-DNA hybridization to test for the presence of 36 species. All of the species investigated were detected in both groups, however there were differences in proportions of species. Four species were detected at significantly higher levels in peri-implantitis group supragingivally, which were *P. gingivalis, T. forsythia, T. denticola* and *Prevotella nigrescens* (P < 0.05).
Subgingivally, the levels of *P. gingivalis, T. forsythia, T. denticola, F. nucleatum ss. nucleatum, F. nucleatum ss. vincentii* and *P. intermedia* were significantly higher in implants with peri-implantitis (P < 0.05) suggesting that organisms commonly associated with periodontal diseases could also be important in peri-implantitis.

In an attempt to correlate, clinical indices with histological and microbiological findings, Ferreira et al., (Ferreira et al., 2009) used PCR and species-specific probes to check for the presence of *A. actinomycetemcomitans, P. gingivalis,* and *T. forsythia* in the subgingival plaque around 10 implants that ‘were indicated for treatment of the peri-implant pockets’. They failed to find statistically significant correlations between the presence of these bacteria and the degree of inflammation.

In summary, the peri-implant microbial community appears to be complex, however all previous studies have targeted specific organisms or focused on groups of organisms. This undermines the true complexity of this community and may create false associations with the disease.
1.3 Dental Biofilms

Biofilms can be defined as one or more communities of micro-organisms embedded in a polymeric, extracellular substance of glycoproteins and attached to a solid surface (Socransky and Haffajee, 2002)

Biofilms provide protection to the resident bacteria from both host defenses and exogenous antimicrobial treatments. It has been suggested that bacteria in a biofilm are up to 1000 times more resistant to antimicrobials than the same cells growing in liquid culture (Costerton et al., 1999, Mah and O'toole, 2001). This makes infections developing on permanently or temporarily implanted biomaterials such as heart valves, vascular prostheses or dental implants poorly responsive to antimicrobial treatment and generally difficult to manage.

Biofilm formation is a step-wise process, which starts by the adhesion of planktonic or free-floating microorganisms on a surface. This initial adhesion is usually promoted in the oral cavity via receptors provided by the acquired pellicle, a salivary film that coats oral surfaces and contains albumin, glycoproteins, acidic proline-rich proteins, mucins, sialic acid and other components. Early colonizers attach to this pellicle, followed by other steps, which involve colonization, co-aggregation and co-adhesion of secondary/late colonizers and subsequent growth and maturation of the biofilm, and finally detachment of some microorganisms. These can adhere to other regions of the oral cavity and produce other bacterial colonies. A simplified representation of biofilm formation is shown in Figure 1 (Kolenbrander et al., 2010, Kuramitsu et al., 2007).

Quirynen and Bollen (Quirynen and Bollen, 1995) explained that initial adhesion is initiated when bacteria manage to get close enough (50 nm) to a surface by Brownian motion, convective fluid movements or active locomotion. This is accomplished via the long-range Van der Waal attractive forces, which in turn are opposed by electrostatic repulsive forces. The resultant net force will cause the bacteria to establish an initial reversible weak binding at a distance of 10 to 15 nm from the surface. After initial adhesion, a firmer anchorage can be achieved by extracellular filamentous appendages, by direct contact, or by specific interactions (covalent, ionic or hydrogen bonding). Once established, the firmly attached microorganisms start growing and biofilms develop.
In the case of complex biofilms, it is not simply the presence of a single organism in a complex community which determines the properties of a biofilm, but it is the interactions between the biofilm residents which is crucial. Dental plaque is a classical example of a complex biofilm and it is not merely the sum of its bacterial components that make up its properties. Instead, a sophisticated microbial community is established resulting in novel functions that are essential for biofilm architecture and microbial physiology (Marsh, 1994, Marsh, 2005). For example, through the inter-species mechanism of protection, fastidious anaerobes are able to colonize supposedly aerobic environments (Socransky and Haffajee, 2002).

Accumulation of multispecies communities such as dental plaque may lead to polymicrobial infections. Periodontitis, and similarly peri-implantitis represent one of the best documented polymicrobial infections. The clinician’s management of such diseases becomes unfortunately fraught with uncertainty.
1.3.1 Dental Implant Surfaces as Substrates for Biofilm Formation

Dental implants are mostly made of titanium or titanium alloys. Titanium is a highly biocompatible metal. It is lightweight and durable, having the highest strength-to-weight ratio of any metal. It has the ability of forming an oxide layer (2 – 10 nm thick, usually TiO₂) on its surface within seconds of being exposed to air. This oxide layer is extremely adherent and provides titanium with many desirable properties such as corrosion resistance and chemical stability (Palmquist et al., 2010a, Lautenschlager and Monaghan, 1993).

These titanium implant surfaces, which have micro-surface roughness, provide an ideal biological niche for the development of a plaque biofilm. However, it must be borne in mind that surface roughness is only one of the parameters involved in plaque formation. Bacterial adherence is a complicated process, and involves the interplay of several factors. These include other surface characteristics such as chemical composition, surface energy, surface wettability, and surface topography as well as the surrounding environment (saliva, gingival crevicular fluid or culture media) (Ichikawa et al., 1998, Dorkhan et al., 2012, Almaguer-Flores et al., 2012) and bacterial composition. These factors act together to provide distinct patterns for protein adsorption (salivary pellicle) and subsequent bacterial binding (An and Friedman, 1998).

During the past two decades, there has been considerable focus on the optimization of the physico-chemical and mechanical properties of implant materials. Different surface topographies with various chemical compositions have been developed aiming at enhancing osseointegration and increasing clinical success rates. The methods used to develop these surfaces include sandblasting, acid etching, titanium plasma spraying, plasma nitriding and the use of different chemical coatings. A major concern, however still lies in the accumulation of bacteria on these surfaces. Thus, surface optimization must also take in consideration the importance of minimizing bacterial adhesion to avoid development and progression of peri-implant disease. The effect of implant surfaces on bone cells will be discussed in more detail in the last part of this study. The following review will focus on titanium surface characteristics that affect the accumulation of a plaque biofilm.
The Importance of Different Surface Parameters That Affect Biofilm Formation on Titanium Surfaces

1.3.2.1 Surface Roughness

A particularly important structural property of oral implants is the surface topography, or surface roughness. Most currently available implant surfaces are minimally or moderately rough with an average deviation of 0.5 – 2 µm (Albrektsson and Wennerberg, 2004a). Recently, interest has been directed at providing modifications at the nanometer level. Nanosurfaces are those with structures smaller than 100 nm in at least 1 dimension. At least four commercially available implants are presently characterized as having nanofeatures (Wennerberg and Albrektsson, 2010).

Although many studies have been conducted assessing the effect of titanium surface roughness on biofilm formation, there is some controversy about the role of surface roughness and plaque accumulation.

Studies Which Support That Increasing Surface Roughness Increases Biofilm Formation

a) In vivo Studies

Generally, it has been considered that the rougher the surface, the more biofilm is formed. From a series of split mouth studies, Bollen and Quirynen observed a positive correlation between plaque accumulation and surface roughness on different materials tested intraorally (Quirynen et al., 1990). This was less obvious in patients with optimal oral hygiene (Quirynen et al., 2002). After several days of undisturbed plaque formation, the rougher (Ra =0.8 and Ra =2.1um) surfaces not only seemed to harbour more plaque compared to the smooth surfaces (Ra =0.35 and 0.1um), but also a more pathogenic flora (i.e. spirochaetes and motile organisms) (Quirynen et al., 1993, Quirynen et al., 1990). This was similar for implant abutments, where rougher implant abutments harboured 25 times more subgingival biofilm than their smoother counterparts (Quirynen et al., 1993).

Their explanation for this was that bacteria found it easier to accumulate on these rougher surfaces because at these locations, bacteria were sheltered from removal forces and had the time to establish a less reversible and stronger attachment. Moreover the increased surface roughness provided a greater surface area available for colonization. They also suggested that the significance of surface roughness was less important in
subgingival areas since shear removal forces are less in these locations making the environment more favourable for undisrupted plaque accumulation (Quirynen et al., 1996a, Bollen et al., 1997).

In this context, Nyvad and Fejerskov (Nyvad and Fejerskov, 1987) using scanning electron microscopy revealed that initial colonization started at the depths of surface irregularities, like pits and grooves making it more difficult to completely remove bacteria from these regions.

Studies by other authors also support the theory by Bollen and Quirynen, that increasing surface roughness increases biofilm formation. Amarante et al. (Amarante et al., 2008) demonstrated that titanium plasma sprayed (TPS) rough surfaces accumulated more plaque, and showed that they were more difficult to clean by tooth brushing. Conversely, Burgers et al. (Burgers et al., 2010) compared 2 surfaces with significant differences in surface roughness. One had a microrough sand blasted acid etched (SLA) surface and the other a nanorough (machined titanium) surface from Camlog implants (Camlog Biotechnologies AG, Basel, Switzerland). They tested the surfaces both in vivo and in vitro using two fluorometric techniques (Resazurin and live/dead staining) and confirmed that surface roughness did indeed cause a greater accumulation of biofilm. This phenomenon was even more pronounced in in vivo testing probably because of the sheltering effect of rough surfaces against removal forces, which did not occur under semi-static in vitro conditions (Rimondini et al., 1997).

Bollen et al., (Bollen et al., 1996, Quirynen et al., 1996a, Bollen et al., 1997) demonstrated that abutment smoothening below Ra of 0.2 µm showed no further reduction in the resultant biofilm. Thus an Ra value of 0.2 µm was proposed, and has been generally accepted, to be the average roughness threshold below which bacterial adhesion is not affected. They attributed this to the size of oral bacteria, which range in size from 0.5 µm to 10 µm. Thus the mean peak to valley heights (Rtm) and mean spaces between peaks (Sm) present in surfaces with an Ra of 0.2 µm or less, would be too small to offer any major sheltering possibilities and would not be able to protect these bacteria against removal forces (Quirynen et al., 1996a, Bollen et al., 1997).

However, judging from the results of other studies in the literature, the proposed threshold value of 0.2 µm may be questionable. Rimondini et al. (Rimondini et al., 1997) detected differences in the adherent biofilm between smoothest (Ra 0.088 µm)
and roughest surfaces (Ra 2.142 µm), but not between surfaces with an Ra 0.088 µm and Ra 0.2 µm nor between surfaces of Ra 0.2 µm and Ra 2.142 µm. Similarly, Nakazato et al. (Nakazato et al., 1989) did not detect any significant reduction of biofilm on surfaces with an Ra less than 0.37 µm questioning the 0.2 µm threshold value.

c) **In vitro studies**

An *in vitro* study demonstrated that surface roughness had a strong influence on plaque accumulation, where an exponential increase in the attachment of *S. sanguinis* was observed after 7 days on increasing surface roughness from Ra 0.17 µm to 1.14 µm to 3.17 µm (Pereira da Silva et al., 2005).

Similarly, in another study, colonization by the same bacterium was shown to be significantly different after 24 hours on titanium discs polished with 1 µm diamond paste (7x10⁴ CFU/mm²), 800-grit silicon carbide sandpaper (2 x 10⁶ CFU/mm²), or sandblasted (1x10⁶ CFU/mm²), however the surface roughness produced by these methods was not reported (Drake et al., 1999).

**STUDIES WHICH DO NOT SUPPORT THAT INCREASING SURFACE ROUGHNESS INCREASES BIOFILM FORMATION**

Even so, many other studies report that roughness is only a minor factor and do not support the relationship between biofilm formation and different implant surface roughness.

a) **In vivo studies**

An *in vivo* pilot study on 3 patients using implant surfaces from Straumann (Institut Straumann AG, Basel, Switzerland) showed that changes in microtopography had a highly uneven and unpredictable influence on supragingival plaque accumulation. On one hand, surface roughness of Ra 3.2 µm were covered with significantly more plaque than that on surfaces with Ra of 0.83 µm. On the other hand, the most polished group (Ra = 0.04 µm) revealed significantly higher plaque biofilm coverage after 12, 24 and 48 hours compared to surfaces with a Ra of 0.83 µm (Schwarz et al., 2007b).

Similarly, in an *in vivo* study in which implant abutments (Nobel Biocare, Goteborg, Sweden) were worn for 14 days, no influence of surface roughness could be detected in the subgingival areas although supragingivally bacterial adhesion was found to be
greater on rougher surfaces (Elter et al., 2008). This was contrary to the subgingival findings by Quirynen et al. mentioned in the previous section (Quirynen et al., 1993).

In accordance with the above, analyses of the results from the biofilm produced on titanium discs embedded in intraoral splints worn by 12 volunteers failed to detect a significant difference in bacterial adhesion between the two titanium surfaces used in this study, although one of the surfaces, TiUnite® (Nobel Biocare, Gothenburg, Sweden) was 10 times rougher (Ra = 544 nm) than the machined titanium surface (Ra = 54 nm) also from Nobel Biocare (Nobel Biocare, Gothenburg, Sweden). Using molecular probes, FISH and confocal examination, streptococcus species were shown to be the main component of the biofilm (Al-Ahmad et al., 2010).

b) In vitro studies

These results were confirmed by various in vitro studies. One study, using a three species bacterial consortium made of A. naeslundii, S. sanguinis and Lactobacillus salivarius, bacterial adhesion was found to be less on a rougher surface (Ra = 1.52 μm) when compared to a smoother surface (Ra = 0.22 μm) after 14h. However these surfaces were also different regarding their chemical composition (blasted titanium and calcium-modified titanium surfaces respectively), and this supports that roughness is not the only contributing factor (Frojd et al., 2011a).

Another short term in vitro study (Schmidlin et al., 2013) showed that regardless of a titanium’s surface roughness, bacterial colonization was quite comparable. Biofilms were made up of 6 species (Actinomyces oris, Veillonella dispar, F. nucleatum, Streptococcus oralis, Streptococcus sobrinus, and Candida albicans) and were grown on Straumann implant surfaces (Institut Straumann AG, Basel, Switzerland) with 4 different degrees of roughness (Ra was 0.1μm, 0.3 μm, 0.6 μm and 3.2 μm). There were minute insignificant differences that remained constant over time (at 20 minutes, 2 hours, 8 hours and 16 hours).

Likewise, another short term in vitro study which used 9 species representing the various complexes of subgingival plaque (Actinomyces israelii, A. actinomycetemcomitans, C. rectus, E. corrodens, F. nucleatum, Parvimonas micra, P. gingivalis, P. intermedia, S. sanguinis) grown on three Straumann surfaces (Ra < 0.2, Ra = 0.8, Ra = 3.2) for 24 hours, revealed no consistent pattern indicating that rougher surfaces was associated with more bacterial adhesion (Almaguer-Flores et al., 2012).
This was confirmed using resazurin metabolism assay and sonication-colony-forming unit counts to assess biofilm growth on two distinctly different surfaces in terms of roughness level (Sa 0.3 μm vs. 1.4 μm). Again no difference in biofilm growth could be detected when comparing both surfaces at days 1 and 3 (Lin et al., 2013).

In the first part of the study by Duarte et al., a greater bacterial adhesion of *S. sanguinis* was exhibited on rough surfaces (Ra = 0.7 μm) compared to smooth ones (Ra = 0.2 μm) after incubation for 16 hours. However, after these surfaces were cleaned with various treatment methods and their roughness values changed (the air-powder cleaning system did not affect the surface roughness whereas the metal curettes made the smooth surfaces rougher), the resultant biofilms formed were similar on smooth and rough surfaces irrespective of treatment modality and roughness value. Thus it appears that the level of bacterial adhesion is affected by other factors in addition to surface roughness (Duarte et al., 2009b).

Yoshinari et al., Almaguer-Flores et al., Schwarz et al. and Al-Ahmad et al. also reported more controversial findings where the influence of surface roughness was unpredictable in their studies (Yoshinari et al., 2000, Almaguer-Flores et al., 2012, Schwarz et al., 2007a, Al-Ahmad et al., 2010).

A summary of *in vitro* and *in vivo* studies conducted on biofilm formation on dental implant surfaces can be found in tables 3 and 4.

1.3.2.2 Surface Free Energy and Surface Wettability

Surface free energy (SFE) and Surface Wettability (Contact angle measurements) are proposed to be two other fundamental properties to the process of microbial adhesion. They will be discussed together since SFE is mostly calculated indirectly using contact angle measurements.

The importance of SFE can be explained by the thermodynamic model of microbial adhesion which states that bacterial strains with a high SFE preferentially adhere to surfaces with high SFE because the bacteria have a negative interfacial free energy of adhesion (Weerkamp et al., 1985, Burgers et al., 2010). The wicking effect of hydrophilic surfaces on tissue fluids has been shown to enhance the absorption of cell attachment proteins like fibronectin which may at the same time provide binding sites for bacteria and in turn increase bacterial attraction (Almaguer-Flores et al., 2012).
The methods of altering SFE and wettability are similar to the methods used to alter surface roughness and hence a dichotomy appears to exist regarding the relative importance of each.

Several studies have shown that SFE and surface wettability were the most prominent factors, while surface roughness was less important. For example, an increased biofilm thickness was observed on machined titanium, which although much smoother than many of the other substrates used, was hydrophobic in comparison to the other hydrophilic surfaces suggesting that surface wetting properties and SFE both play important roles (Al-Ahmad et al., 2010). Moreover, Al-Radha et al. (Al-Radha et al., 2012) suggested that the influence of SFE was the most important factor determining initial bacterial adhesion to a smooth surface where a decrease in SFE from 37.58 mN/m to 33.4 mN/m led to a decrease in the area covered by S. mitis from 7.38% to 3.13% when using saliva as the culture media. Another study by Drake et al., (Drake et al., 1999) reported that when the SFE of dental implants of varying surface roughness was increased and the surfaces made more wettable; these surfaces showed a significant decrease in bacterial adhesion than the same surfaces that were hydrophobic (less wettable) and had lower SFE. Although effects of this latter study are opposite to what the previous studies had indicated, it still points out that SFE and wettability were the major determining surface characteristics.

On the contrary, most studies suggest that roughness is the prominent parameter and overrules the effect of SFE and wettability (Nakazato et al., 1989, Quirynen and Bollen, 1995, Burgers et al., 2010, Schwarz et al., 2007a, Quirynen et al., 1990).

For instance, ultrahydrophilic surfaces with increased SFE (SLActive® Institut Straumann AG, Basel, Switzerland) showed slightly less bacterial adhesion at 12, 24 and 48 hours compared to their non-modified counterparts (SLA® Institut Straumann AG, Basel, Switzerland). However this difference was not significant and they concluded that hydrophilicity had no influence on supragingival plaque formation (Schwarz et al., 2007a).

The same surfaces used in another study by different authors showed the opposite where the ultrahydrophilic surfaces showed slightly more bacterial adhesion than the non-modified surfaces at 20 minutes, 4 hours, 8 hours, and 16.5 hours. However, this was not significant and the authors came to the same conclusion that bacterial colonization
was quite similar on all implant materials over time regardless of their wetting and SFE properties (Schmidlin et al., 2013).

A third group using the same surfaces confirmed that hydrophilicity and surface energy could not explain the differences in bacterial adhesion. The hydrophilic surfaces showed a minute increase in bacterial adhesion that was insignificant and inconsistent (Almaguer-Flores, Olivares-Navarrete et al. 2012).

These findings were consistent with an in vivo study by Elter et al., where the influence of acid passivation and increased hydrophilicity did not play a key role in biofilm formation (Elter et al., 2008).

In another study, surfaces that had significantly higher SFE (39.6 mj/m²) had significantly less microbial adhesion than surfaces with a lower SFE (18.1 mj/m²) both in vivo and in vitro. This supports the predominant influence of Ra in contrast to SFE (Burgers et al., 2010).

Finally, Yoshinari et al. (2000) showed a weak and uneven importance for SFE. They indicated that it might selectively influence certain species such as *P. gingivalis* which showed a positive but weak correlation, whereas others such as *A. actinomycetemcomitans* were not correlated to SFE (Yoshinari et al., 2000).

1.3.2.3 Biochemical Modifications

Another interesting approach to improve osseointegration and enhance bone healing is to incorporate and/ or attach molecules and pharmacological substances for local release from the implant surface (biochemical modification). This is a promising area for the development of future oral implants for use in compromised clinical situations. Such modifications should however be tested to ensure they do not promote the accumulation of bacteria.

Calcium incorporated on the surface of oral implants was found to decrease bacterial adhesion after two hours of incubation, but after 14 hours, this effect was reversed and implant surfaces with calcium on them seemed to attract more bacteria (Frojd et al., 2011b), even though the surfaces were considerably smoother (surfaces with Ca²⁺ had an Ra = 0.22 µm vs. blasted surfaces with an Ra = 1.52 µm). In another study (Badihi Hauslich et al., 2013), pretreatment of surfaces with calcium ions increased the adhesion of *Streptococcus mutans* and *F. nucleatum* to titanium, but had no influence on *P.
gingivalis, while research using an in vitro model system and single species cultures, concluded that the incorporation of calcium ions in titanium increased the adhesion of late colonizers such as P. gingivalis and A. actinomycetemcomitans. In the same study, titanium implanted with fluoride ions significantly inhibited the growth of both P. gingivalis and A. actinomycetemcomitans when compared with the polished commercially pure titanium specimens (Yoshinari et al., 2001).

When the effect of different proteins of the conditioning pellicle were tested, it was shown that albumin significantly reduced the adherence of gram positive S. mutans, but had no influence on the adherence of gram negative F. nucleatum and P. gingivalis, whilst fibronectin significantly increased adherence of F. nucleatum and P. gingivalis, but had no significant effect on the adherence of S. mutans, pointing to the fact that different components of the conditioning film might act differently on specific oral bacteria and verifying the complexity of the interactions between serum constituents, oral bacteria and titanium surface properties (Badihi Hauslich et al., 2013).

1.3.2.4 Sterilization method

Even sterilization methods have an effect on surface behaviour in relation to bacterial colonization. Surfaces sterilized by ethylene oxide or by plasma cleaning using argon gas both showed significantly reduced bacterial adhesion. The authors attributed this to the residual effect of the biocidal elements of ethylene oxide gas and the highly wettable, contaminant-free surface produced using argon gas. On the other hand, titanium discs that were steam autoclaved multiple times showed significantly greater bacterial adhesion (regardless of surface roughness). In this case, it was explained that this might have been due to the fact that steam autoclaving increased surface oxide thickness and caused contamination with various elements like carbon, nitrogen, calcium, sodium, potassium and iron. This decreased surface energy and increased hydrophobicity, hence increased bacterial colonization (Drake et al., 1999).
Table 3 Summary of *in vitro* studies of biofilms on implant surfaces (sorted according to incubation time).

<table>
<thead>
<tr>
<th>Authors</th>
<th>Surfaces tested (Roughness value Ra except if stated)</th>
<th>Incubation time and bacteria used</th>
<th>Evaluation method</th>
<th>Results (biofilm accumulation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Schmidlin et al., 2013)</td>
<td>1) Machined (Sa 0.1µm) 2) Machined (Sa 0.3µm) 3) Stained (Sa 0.4µm) 4) Acid-etched (Sa 0.6µm) 5) Sandblasted/ acid-etched (SLA) (Sa 1.3µm) 6) Modified acid-etched 7) Modified SLA</td>
<td>20 minutes 2 hours 4 hours 8 hours 16 hours A. <em>oris</em>, V. <em>dispar</em>, F. <em>nucleatum</em>, S. <em>oralis</em>, S. <em>sobrinus</em>, C. <em>albicans</em></td>
<td>Culture (Non-selective and selective)</td>
<td>Roughness and wettability did not influence biofilm formation (which increased over time)</td>
</tr>
</tbody>
</table>
| (Yoshinari et al., 2000)       | 1) Polished Titanium (0.07 µm) 2) Blasted Ti (0.47 µm) 3) Striated Ti (0.72 µm) 4) + Calcium (0.1 µm) 5) + Fluoride (0.08 µm) 6) Titania sprayed (3.79 µm) 7) Alumina coated (0.06 µm) + several other modifications beyond the scope of this thesis | 1 hour A. *actinomyces-tecumcomitans* (Aa), P. *gingivalis* (Pg) | Direct scintillation counting of labeled bacteria                                                   | Roughness had unpredictable influence.  
- Some correlation of *Pg* (but not *Aa*) with SFE.  
- **Blasted** ↑ *Aa* and ↑ *Pg* than polished  
- **Striated** ↑*Aa* than polished, but ↓ than blasted (*Pg* same as polished)  
- **Calcium**: ↑*Pg* and *Aa*  
- **Fluoride**: No difference  
- **Titania sprayed**: ↓*Pg* (although Ra was very high) and ↑*Aa* than polished  
- **Alumina coated**: ↓*Pg* (no effect on *Aa*) |
| (Burgers et al., 2010) | Pure titanium (0.15µm)  
Textured titanium (0.95 µm) | 2 hours  
* S. sanguinis | 1) Alamar blue/ 
Resazurin  
2) CLSM | 1) 1.4x more on rougher surface  
(resazurin)  
2) 3.7x more on rougher surface  
(CLSM) |
|---|---|---|---|---|
| (Frojd et al., 2011a) | 1) Turned titanium (Sa 0.18 µm)  
2) Sol-gel nanoporous (Sa 0.16 µm)  
3) Anodized Ca++ (Sa 0.22µm) | 2 hours &  
14 hours  
* S. sanguinis &  
* A. naeslundii | 16S rRNA fluorescence in situ hybridization and confocal laser scanning microscopy. | No differences in adhesion between all relatively smooth surfaces at 2 or 14 hours |
| (Frojd et al., 2011b) | 1) Turned (Sa 0.18 µm)  
2) Anodized (Sa 0.4 µm)  
3) Anodized + Calcium acetate (Sa 0.22 µm)  
4) Blasted Al₂O₃ (1.52 µm)  
5) Blasted + anodized + Ca++ acetate (1.54 µm) | 2 hours &  
14 hours  
**2 species model**  
* S. sanguinis,  
* A. naeslundii | RNA fluorescence in situ hybridization and confocal laser scanning microscopy | - At 2 hours rougher surfaces increased biofilm  
- At 14 hours surface roughness had no effect.  
- Calcium had inconsistent effects.  
- The species model used affected results |
| (Al-Radha et al., 2012) | 1) Polished titanium (0.043 µm)  
2) Polished zirconia (0.038 µm)  
3) Ti blasted with zirconia (0.158 µm)  
4) Ti blasted w zirconia + acid etched (0.15µm) | 6 hours  
* S. mitis and  
* P. nigrescens | CLSM | SFE plays a major role in determining biofilm formation on smooth surfaces at early stages when using saliva as the medium. |
<table>
<thead>
<tr>
<th>(Duarte et al., 2009b)</th>
<th><strong>Before treatment:</strong> Smooth (0.2 µm)</th>
<th><strong>After treatment</strong> (metal curettes / air-powder system): Smooth (0.38 µm/ 0.2 µm) SLA (0.73 µm/ 0.7 µm)</th>
<th>16 hours</th>
<th>Culture</th>
<th>Controversial. Before treatment rougher surfaces had more biofilm. After treatment, all surfaces had the same biofilm irrespective of roughness</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Badihi Hauslich et al., 2013)</td>
<td>1) Machined (turned) 2) Dual acid-etched 3) Acid etched/ sandblasted +/- Coating by human serum albumin or fibronectin +/- Pretreatment with Ca^{++} or K</td>
<td>Overnight</td>
<td>SEM CLSM DNA analysis</td>
<td><strong>Rougher</strong> surfaces had more biofilm. <strong>Albumin</strong> ↓S.m (no effect on P.g or F.n) <strong>Fibronectin</strong> ↑ P. g and F.n (no effect on S.m) <strong>Calcium</strong> ↑ S.m and F.n (no effect on P.g) <strong>Potassium</strong> No effect</td>
<td></td>
</tr>
<tr>
<td>(Almaguer- Flores et al., 2012)</td>
<td>1) Grade II titanium (Sa &lt; 0.2µm) 2) Acid-etched (Sa 0.8µm) 3) Modified (hydrophilic) acid-etched 4) Sandblasted/ acid-etched (SLA) (Sa 3.2) 5) Modified (hydrophilic) SLA</td>
<td>24 hours</td>
<td>Checkerboard DNA-DNA hybridization technique</td>
<td>Differences in biofilm could not be attributed to a single factor.</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>S. sanguinis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study (Year)</td>
<td>Surface Treatments</td>
<td>Time Points</td>
<td>Methods</td>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------</td>
<td>-------------</td>
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<td>---------</td>
<td></td>
</tr>
<tr>
<td>Lin et al., 2013</td>
<td>1) Pickled Ti surface (Sa 0.3 µm) 2) Sandblasted acid etched (Sa 1.4 µm)</td>
<td>1 day &amp; 3 days</td>
<td>S. mutans/ P. gingivalis</td>
<td>1) Resazurin 2) Culture</td>
<td>Surface roughness had no influence on amount of biofilm formed at either time points.</td>
</tr>
<tr>
<td>Yoshinari et al., 2001</td>
<td>Same as their previous study</td>
<td>48 hours</td>
<td>Aa / Pg</td>
<td>Antibacterial activity test (culture)</td>
<td>Only fluoride implanted specimens exhibited antibacterial activity against both Aa and Pg</td>
</tr>
</tbody>
</table>
| Drake et al., 1999 | **3 Roughness Values**  
   a) Diamond paste 1µm  
   b) 800 grit silicon carbide sandpaper  
   c) Sandblasted Acid  
   a) Acid passivated  
   b) Non-acid passivated  
   **Sterilization**  
   a) UV light  
   b) Steam autoclaving  
   c) Ethylene oxide gas  
   d) Plasma cleaning | 72 hours | S. sanguinis | Culture | - Greater adhesion on rougher surfaces.  
- Less adhesion on hydrophilic surfaces.  
- Autoclaving increased biofilm on all surfaces.  
- Effect of hydrophilicity was more important than roughness |
| Pereira da Silva et al., 2005 | 1) Machined titanium (0.17 µm)  
2) Blasted titanium (1.14 µm)  
3) Blasted titanium (3.17 µm) | 7 days | S. sanguinis | Culture | Biofilm directly proportional to roughness |
Table 4 Summary of *in vivo* studies of biofilms on implant surfaces (sorted according to incubation time)

<table>
<thead>
<tr>
<th>Author</th>
<th>Surfaces tested (Roughness value Ra except if stated)</th>
<th>Duration</th>
<th>Method</th>
<th>Results</th>
</tr>
</thead>
</table>
| (Nakazato et al., 1989)    | 1) Single crystal alumina (0.09µm)  
2) Polycrystal alumina (0.854µm)  
3) Partially stabilized zirconia (0.369µm)  
4) Hydroxyapatite (0.518µm)  
5) Pure titanium (0.142µm)  
6) Acrylic resin (0.109µm) | 4 hours  
48 hours | SEM  
Culture                           | At 4 hours biofilm formation tended to be affected by surface roughness rather than surface free energy.  
At 48 hours there was no significant difference. |
| (Schwarz et al., 2007a)    | a) Polished (0.04 µm)  
b) Acid-etched (0.83 µm)  
c) Modified acid etched  
d) Sandblasted acid etched (3.22 µm)  
e) Modified SLA | 12 hours  
24 hours  
48 hours | Erythrosine dye stain + digital camera | Hydrophilicity had no effect, while microtopography had an uneven and unpredictable influence |
| (Burgers et al., 2010)     | 1) Pure titanium (0.15 µm)  
2) Textured titanium (0.95 µm) | 12 hours | 1) Alamar blue/Resazurin  
2) CLSM                                   | 7.7 fold biofilm increase on rougher surfaces |
| (Rimondini et al., 1997)   | a) 0.088 µm  
b) 0.2 µm  
c) 2.142 µm | 24 hours | 1) MTT  
2) SEM                                      | More biofilm on roughest (2.1) compared to the smoothest (0.088), but not when groups a) & b) were compared or when b) and c) were compared |
| (Rasperini et al., 1998)   | 1) Titanium  
2) Ceramic | 6 hours  
24 hours  
7 days  
14 days | 1) Luciferase (ATP analysis) assay  
2) Direct bacteria count                  | No differences in bacterial adhesion.  
Maximum amount of colonization was achieved after 24 h and counts remained constant for the rest of the 14 days |
<table>
<thead>
<tr>
<th>Study</th>
<th>Surfaces</th>
<th>Time Points</th>
<th>Methods</th>
<th>Observations</th>
</tr>
</thead>
</table>
| Al-Ahmad et al., 2010  | 1) Machined titanium (54nm)  
2) Modified titanium (TiUnite) (544nm)  
3) Modified zirconia (ZiUnite) (488nm)  
4) Machined alumina-toughened zirconia (21nm)  
5) Sandblasted alumina-toughened zirconia (275nm)  
6) Machined zirconia (129nm)  
7) Bovine enamel (14nm) | 3 days  
5 days | FISH and CLSM | With the exception of machined titanium (hydrophobic), biofilm was correlated with surface roughness at 3 days.  
At 5 days \(\rightarrow\) no differences.  
Streptococci were the main component of the biofilm |
| Elter et al., 2008     | 1) Untreated titanium (0.2 \(\mu\)m)  
2) Ground (0.3 \(\mu\)m)  
3) Acid etched (0.4 \(\mu\)m)  
4) Sandblasted (0.9 \(\mu\)m) | 14 days | SEM | Controversial: Supragingivally sandblasted more biofilm than the rest, but no difference between the other groups. Subgingivally no difference between any. |
| Quirynen et al., 1993  | 1) Standard abutment (0.35 \(\mu\)m)  
2) Roughened titanium abutment (0.8 \(\mu\)m) | 3 months | Differential phase-contrast microscopy & DNA probe analysis & culturing | When plaque was collected with paper points, only minor differences were found.  
When the microbiota adhering to the abutment were considered, rough surfaces harboured 25 times more bacteria |
<table>
<thead>
<tr>
<th>Study (Quirynen et al., 1996a)</th>
<th>Abutment Types</th>
<th>Surface Roughnesses</th>
<th>Time Points</th>
<th>Testing Methods</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Standard Brånemark abutment (0.21 µm) with a relatively smooth surface</td>
<td></td>
<td></td>
<td>3 months</td>
<td>Culture DPCM</td>
<td>No difference (quantitatively or qualitatively) between plaque build up, supra (curettes) or subgingivally (paper points).</td>
</tr>
<tr>
<td>2) A machine-polished abutment (0.11 µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) A machine-polished and electropolished abutment (0.13 µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4) A manually polished abutment (0.05 µm)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Study (Bollen et al., 1996)</th>
<th>Abutment Types</th>
<th>Surface Roughnesses</th>
<th>Time Points</th>
<th>Testing Methods</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overdenture abutments</td>
<td>Titanium smooth (0.2 µm)</td>
<td></td>
<td>3 months</td>
<td>DPCM Culture</td>
<td>No difference (ultrasmooth harboured slightly more supragingivally)</td>
</tr>
<tr>
<td>- Ceramic ultrasmooth (0.06 µm)</td>
<td></td>
<td>12 months</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
1.3.3 Implant Surfaces and peri-implant diseases

The limited data available does not show any evidence that a particular surface has a greater risk of developing peri-implantitis.

1.3.3.1 Human Studies

A retrospective controlled trial comparing turned machined (Brånemark) surfaces and rough TPS (Straumann) surfaces showed an increased risk of peri-implantitis for TPS implants in comparison to turned implants. Peri-implantitis was seen in seven out of 77 TPS implants, one of which subsequently failed completely at 12 months and another at three years (Astrand et al., 2004a). However other studies failed to detect an increased risk of peri-implantitis for rougher surfaces (Astra TiOblast and Biomet 3i fully dual acid etched) when compared to smoother turned surfaces (Astra turned and Biomet hybrid dual acid etched respectively) (Astrand et al., 2004a, Zetterqvist et al., 2010).

1.3.4 Animal Studies (Ligature-induced peri-implantitis)

All existing animal studies have used the ligature-induced peri-implantitis model in dogs. The design of these studies has changed after 2008 so that the hard tissue destruction that occurs after, not before the “active breakdown phase induced by the ligatures” is considered the starting point of the observation period. The “spontaneous progression” and plaque accumulation that occurs from thereon (plaque accumulation period) is considered a more valid representation of the naturally occurring peri-implantitis (Lang et al., 2011).

Out of four implant surfaces tested in dogs (Nobel TiUnite, Astra TiOblast, Biomet 3i turned, Straumann SLA), progression of peri-implantitis was significantly more pronounced clinically and histologically on the TiUnite surface, which is an anodized moderately rough surface (Albouy et al., 2008). In another study, there was increased bone loss radiographically and inflammatory cell infiltrate histologically with SLA surfaces compared to turned surfaces (both Straumann) suggesting a greater risk of peri-implantitis around rougher surfaces (Berglundh et al., 2007b).
1.4 Treatment of Peri-Implantitis

Based on our understanding about the aetiopathogenesis of peri-implantitis, different treatment methods have been proposed. Most of these are empirical recommendations and there is limited evidence to suggest superiority of one treatment modality over the other. However the general agreement is that it is essential to remove the plaque biofilm and maintain a biofilm-free surface (Subramani and Wismeijer, 2012).

The methods that have been proposed to achieve this include:

- Mechanical debridement (curettes, ultrasonic devices, air-polishing devices)
- LASER treatment (e.g. CO2, ER:YAG)
- The use of chemical and antimicrobial agents (eg. chlorhexidine, hydrogen peroxide, citric acid, local and systemic antibiotic therapy)
- Surgical procedures. These involve open flap debridement to enable improved access to the implant surface in order to debride it effectively (using one of the methods mentioned above) and establish a plaque-free surface. Surgical procedures are usually accompanied by tissue resective or bone regenerative procedures in order to facilitate improved oral hygiene for the patient.

In addition the following factors have been included:

- Smoothening the implant surface to eliminate microbial retentive factors (implantoplasty) (Romeo et al., 2007).
- Adjusting, if necessary, the prosthesis to allow proper oral hygiene.
- Reinforcement of oral hygiene instructions, continuous maintenance and supportive peri-implant therapy.

In many cases, more than one of these methods have been combined and the amount of bone regeneration and re-osseointegration has varied considerably between studies.
The cumulative interceptive supportive therapy (CIST) is a strategy that has been proposed to manage peri-implant diseases (Figure 2). This protocol relies on periodic assessment of probing pocket depth (PPD), bleeding on probing (BOP), presence of suppuration, and, if indicated, radiographic evaluation. It includes four treatment modalities: A = mechanical debridement; B = antiseptic treatment; C = antibiotic treatment; and D = regenerative or resective surgery and is cumulative in nature, meaning that according to the score obtained from the diagnosis, the patient receives the treatment for that score in addition to those for the previous codes (Lang et al., 2000).

Figure 2 Decision tree for cumulative interceptive supportive therapy (CIST). Depending on the mucosal condition, probing depth and bone loss, either A, A+B, A+B+C, or A+B+C+D is performed. A: Mechanical debridement; B: antiseptic cleaning; C: antibiotic therapy; D: resective or regenerative surgery. Adopted from Lang et al. (Lang et al., 2000).

A helpful set of guidelines for the treatment of peri-implant diseases have also been developed and published by the Association of Dental Implantologists, UK (ADI). The algorithms for the non-surgical and surgical therapeutic approaches can be found in Appendix 1.
1.4.1 Non-surgical treatment of peri-implantitis

The available literature suggests that a non-surgical approach to peri-implantitis, although unpredictable could be beneficial as a first line of treatment before attempting a surgical approach, and various methods have been advocated with no definitive gold standard e.g. manual and ultrasonic debridement, air polishing/abrasion, laser, photodynamic therapy, saline and the application of various medications (e.g. topical antibiotics) and chemical agents (e.g. citric acid, hydrogen peroxide, chlorhexidine, etc.) (Lindhe et al., 2008).

1.4.1.1 Manual debridement

Hand instruments made of titanium or ceramic materials and ultrasonic tips with plastic, teflon or carbon fibre coatings have been specifically developed for the mechanical debridement of dental implants (Renvert and Giovannoli, 2012). Some authors have questioned the efficacy of these different materials while others have advised against using instruments softer than titanium to avoid remnants of the instrument remaining on the implant surface and acting as a foreign body (Augthun et al., 1998).

Furthermore, studies such as those conducted by Karring et al. (Karring et al., 2005) and Renvert et al. (Renvert et al., 2009) concluded that there were no differences in the treatment outcomes clinically and microbiologically using specifically developed tips such as the vector system (an ultrasonic device with a carbon fibre tip combined with an aerosol spray of hydroxyapatite particles) and traditional manual debridement using carbon fiber curettes or titanium curettes respectively.

1.4.1.2 Air – polishing and abrasion

The most commonly used mechanical method to debride implant surfaces and remove the plaque biofilm is to use manual or ultrasonic scalers. However accessibility problems in addition to the inherent difficulties for implant debridement imposed by implant threads and surface roughness may hinder effective cleaning of implants. Air polishing devices (APDs) may overcome some of these difficulties if direct access to the surface cannot be achieved.

These devices have already been shown to be a viable treatment option in periodontal maintenance. In addition, patients have described treatment with APDs as being
significantly more comfortable than scaling with curettes (Moene et al., 2010, Petersilka et al., 2003b, Petersilka et al., 2003c).

1.4.1.3 Laser Therapy

Laser decontamination is based on its thermal effect, which denatures proteins and causes cellular necrosis (Kreisler et al., 2002). Among the lasers employed, the Er:YAG laser is considered to possess the best properties for both degranulation and implant surface decontamination. It has been shown to be effective for biofilm removal, having bactericidal effects, which do not damage implant surfaces (Schwarz et al., 2005b). However clinical studies using lasers for the treatment of peri-implantitis have failed to demonstrate any significant improvement in outcomes when compared to manual debridement (Schwarz et al., 2005c) or to air-polishing devices (Renvert et al., 2011).

1.4.1.4 Photodynamic Therapy

Photodynamic therapy is a technique based on marking the bacterial wall with a light sensitive dye (phenothiazine chloride), which when irradiated with laser (wavelength of 660 nm) produces cytotoxic singlet oxygen, which is able to destroy bacterial cells. Schär et al. (Schar et al., 2013) compared locally administered minocycline microspheres (Arestin) and photodynamic therapy (HELBO) in the treatment of initial peri-implantitis (PPD 4-6 mm) and did not find that using photodynamic therapy achieved any superior results. The initial debridement protocol in both groups involved the use of titanium curettes, glycine-based air-powder abrasion, and irrigation using 3% hydrogen peroxide followed by photodynamic therapy in one group and minocycline in the control group. Both treatments led to clinical improvement with comparable results.

1.4.2 Surgical Treatment of Peri-Implantitis

Limited accessibility and visibility of implant surfaces may in part explain why non-surgical therapy has been associated with limited success, thus access surgery combined with implant surface decontamination is generally advocated in order to achieve complete removal of granulation tissue. Nevertheless, the evidence available for long-term treatment of peri-implantitis using surgical techniques is still limited (Claffey et al., 2008).
1.4.2.1 Access surgery

Maximo, Duarte and de Mendonca (de Mendonca et al., 2009, Duarte et al., 2009a, Maximo et al., 2009) published a series of short-term (3 – 12 months) observations on treatment of peri-implantitis using access surgery without the use of antibiotics. Their decontamination method consisted of mechanical debridement using curettes followed by sodium bicarbonate air abrasion. This resulted in a significant reduction of microbiota, a reduction in TNF alpha levels in the peri-implant crevicular fluid and improved clinical parameters.

1.4.2.2 Apically repositioned flap surgery

A randomized comparative clinical trial (Romeo et al., 2005, Romeo et al., 2007) suggested that resective surgical procedures coupled with implantoplasty could be an effective treatment for rough-surface implants affected by peri-implantitis.

The study by Serino and Turri (Serino and Turri, 2011) also reported on the outcome of a resective surgical procedure that included pocket elimination and bone recontouring. Despite treatment, a total of 36 implants (42%) out of the 86 with initial diagnosis of peri-implantitis presented with peri-implant disease two years later. It was concluded that disease resolution depended on the initial bone loss where implants with no signs of peri-implantitis following treatment tended to remain healthy during the 2-year follow up period, while disease progression was observed for the implants that still showed signs of peri-implant disease following treatment.

1.4.2.3 The adjunctive effects of bone grafts with and without membranes

The consensus report of the sixth EWOP states that currently there seems to be no evidence that ‘regenerative procedures’ [bone grafts/ substitutes, guided bone regeneration (GBR)], performed in implant sites with bone craters, have additional beneficial effects on treatment outcome (Lindhe et al., 2008) and although they may be efficacious for the treatment of peri-implantitis lesions, it must be stressed that such techniques do not address disease resolution, but rather merely fill the osseous defects (Claffey et al., 2008).
The following is a review of case series and randomized controlled trials that have evaluated regenerative treatment of peri-implantitis using a) grafts without membranes and b) grafts with membranes.

**A) WITHOUT MEMBRANES**

Behneke et al. (Behneke et al., 2000) treated 25 implants in 17 patients using an air-powder abrasive, autogenous bone grafts, and systemic metronidazole. A positive result of the reconstructive therapy was observed during a re-evaluation period of up to 3 years. Median marginal bone loss was reduced from 5.3 to 2.3 mm. A radiographic median marginal bone gain of 4 mm was reported at 12 months. Complications occurred in 2 cases and not all implants were followed for the 3 years.

Roccuzzo and co-workers (Roccuzzo et al., 2011) evaluated a regenerative surgical treatment modality for peri-implantitis lesions on two different implant surfaces, TPS and SLA. In 26 patients, implant surfaces were mechanically debrided with a plastic curette and treated using a 24% EDTA gel and a 1% chlorhexidine gel and antibiotics. The bone defect was filled with a bovine-derived xenograft and the flap was sutured around the non-submerged implant. One-year follow-up demonstrated clinical and radiographic improvements in which pocket depths were reduced by 2.1 mm at implants with a TPS surface and 3.4 mm at implants with a SLA surface whereas mean BOP decreased from 91% to 57% (TPS) and from 75% to 15% (SLA). Complete defect fill was not found around TPS surfaces, while it occurred in 3 out of 12 SLA-surface implants. Four of the 26 patients had implants with suppuration and two of them were removed after 12 months.

Wiltfang and colleagues (Wiltfang et al., 2012) studied the effect of regenerative treatment on peri-implantitis in 36 implants using a mixture (1:1) of autologous bone and a demineralized xenograft with growth factors. After resolving the acute infection by local rinsing, granulation tissue was removed. The implants were decontaminated with etching gel and the defects were filled with the bone mix and systemic antibiotics were prescribed. One year evaluation after treatment revealed a mean reduction of 4 mm in PPDs and a mean 3.5 mm gain in bone height. Regarding the implants in which the therapy did not work, 7 implants had PPDs greater than 4 mm, three implants had bone defects greater than 3 mm, and 2 implants were lost due to infection.
**B) WITH MEMBRANES**

The most common complication that was observed when using membranes in the following studies was membrane exposure. The submerged approach was used in half of these studies in order to allow for undisturbed healing and to reduce the risk of infection.

Froum et al. (Froum et al., 2012) evaluated a regenerative approach which included surface decontamination, the use of enamel matrix derivative, a combination of platelet-derived growth factor with anorganic bovine bone or mineralized freeze-dried bone, and finally coverage using a collagen membrane or a subepithelial connective tissue graft. The results from 3 to 7.5 years follow up showed mean P.D. reduction of 5 mm and bone gain of 3 mm. Nevertheless, six out of 38 patients required two to three surgical procedures to achieve the required outcome.

Romanos and Nentwig (Romanos and Nentwig, 2008) used the CO₂ laser to decontaminate failing implants in 15 patients (19 implants). Augmentation with autogenous bone grafting material or xenogenic bone bovine grafting material was used and covered with a collagen membrane. A 3.5 mm mean P.D. reduction was observed and radiographic bone fill varied from partial fill for the autogenous grafted sites to complete fill with the xenograft. Deppe et al. (Deppe et al., 2007) also tested CO₂ laser therapy in a randomized control trial (32 patients, 73 implants) in which conventional decontamination was compared to CO₂ laser decontamination after surgical access was achieved. Augmentation with a mixture of 50:50 autogenous bone and beta tri-calcium phosphate was used and covered with a nonresorbable Gore-Tex membrane. Improved clinical results were reported and laser did not yield additional beneficial effects results compared to conventional decontamination. Conversely, Schwarz and co-workers (Schwarz et al., 2011, Schwarz et al., 2012) compared decontamination using (1) Er:YAG laser and (2) plastic curettes, followed by cotton swabbing with pellets soaked in saline. Following access surgery, granulation tissue removal and implantoplasty, the implants (24 implants, 26 patients) were randomly subjected to the decontamination method chosen and then the intrabony defects were augmented with a bovine xenograft and covered with a collagen membrane. Clinical parameters were recorded at baseline and after 6 and 24 months of non-submerged healing. The decontamination method used for surface debridement seemed to be of subordinate importance since both methods showed comparable clinical improvements. In both groups,
PPD were reduced by an average of 2 mm and BOP sites were reduced from 80% to approximately 30%.

Roos-Jansåker et al. (Roos-Jansaker et al., 2007) augmented the defects using a bone substitute (Algipore®), covered with a resorbable synthetic membrane (Osseoquest) after granulomatous tissue was removed and the implant surface was treated using 3% hydrogen peroxide. Systemic amoxicillin plus metronidazole was prescribed for 10 days and submerged healing was allowed for 6 months. 1 year follow up showed mean reduction of P.D. of 4 mm and bone fill of 2.3 mm.

In a study by Schwarz et al., the healing of intrabony peri-implantitis defects following the application of a nanocrystalline hydroxyapatite or a bovine-derived xenograft in combination with a collagen membrane was evaluated. Results showed that both treatments improved clinical conditions at 6 months (Schwarz et al., 2006a). However, 2 and 4-year published results (Schwarz et al., 2008, Schwarz et al., 2009b) reported greater reductions and clinical attachment level gains in the group treated with xenogenic bone mineral and covered with a collagen membrane. Furthermore, another study by the same authors indicated that circumferential intrabony defects showed greater healing potential than circumferential or semi-circumferential lesions with a buccal dehiscence (Schwarz et al., 2010).

The following two randomized controlled trials have compared surgical techniques that have used bone grafts only to those that have used bone grafting in addition to membranes. They concluded that the addition of membranes did not provide any additional effects.

Khoury and Buchmann (Khoury and Buchmann, 2001) evaluated the outcomes of three regenerative treatment protocols for peri-implantitis. In 25 patients, 41 peri-implant defects were treated with either flap surgery plus autogenous bone graft alone (n = 12); autogenous bone graft plus non-resorbable ePTFE membranes (n = 20); or autogenous bone graft plus resorbable membranes (n = 9) and systemic antimicrobials. Granulomatous tissue was removed and implants were rinsed with 0.2% chlorhexidine gluconate, citric acid, H₂O₂, and 0.9% saline. Three-year radiographic evaluation showed a mean bone gain in all treatment groups. The differences between the three surgical treatment protocols were not significant and the use of membranes was accompanied with more complications.
Similarly, Roos-Jansaker et al. (Roos-Jansaker et al., 2007, Roos-Jansaker et al., 2011) compared using a bone substitute (Algipore®) with a resorbable membrane to using the graft alone to fill the defects. Granulomatous tissue was removed using titanium curettes, the infected implant was cleansed using hydrogen peroxide (3%), followed by profuse rinsing with saline and the patients were ascribed to one of both groups and antimicrobials were prescribed. Again the use of membranes was accompanied with greater complications and did not improve outcome results.

1.4.3 Adjunctive antimicrobials

Although several anti-infective treatment strategies have demonstrated beneficial clinical effects in humans, such as resolution of inflammation, decrease in probing depth and bone gain, there is insufficient evidence to support a specific treatment protocol (Klinge et al., 2002).

In addition, no clinical studies are available on the effects of antibiotics or antiseptic agents as the sole therapy for the treatment of peri-implantitis. In all clinical trials, administration of either antibiotics and/or antiseptics is always combined with either non-surgical or surgical interventions. This leaves the question unanswered as to what extent antibiotics and antiseptic agents, delivered systemically or locally, add to the clinical outcome of the treatment and thus both these adjunctive agents will be considered together in the following review.

1.4.3.1 Non-surgical therapy and antimicrobials

A study by Mombelli et al. (Mombelli and Lang, 1992) documents the clinical and microbiological effects of mechanical debridement in conjunction with systemic antibiotics. After mechanical debridement, peri-implant pockets were irrigated with 0.5% chlorhexidine. Subsequently, systemic ornidazole (1000 mg daily) was administered for 10 days and patients were instructed to irrigate all deep pockets. Significant reduction of PPD, BOP and anaerobic microflora was observed during the 12 months monitoring period and in some lesions, bone fill was also noted.

Büchter et al. (Buchter et al., 2004) compared manual debridement (plastic scaler and submucosal irrigation by chlorhexidine) to the same debridement technique with adjunctive
local delivery of 8.5% doxycycline hyclate gel (Atridox) in patients who lost at least 50% of the bone around implants (i.e. severe forms of peri-implantitis). After 4 months, both groups showed clinical improvements, however mean probing attachment levels improved by 0.61 mm and PPD were reduced by a mean of 0.59 mm in favour of the antibiotic group.

Renvert et al. (Renvert et al., 2004) compared manual debridement using a plastic scaler and application of chlorhexidine gel to debridement using a plastic scaler and local delivery of minocycline HCl (Arestin). They did not find any significant differences between both treatments. They conducted another study (Renvert et al., 2006) in which the same treatments were repeated at 30 and 90 days. Again, no significant differences between groups could be observed, but the antibiotic group consistently showed marginal improvements.

1.4.3.2 Surgical therapy and antimicrobials

In a case series report by Leonhardt et al. (Leonhardt et al., 2003) the long-term outcome of access surgery was evaluated in 9 patients (26 implants). Implant surfaces were decontaminated with H$_2$O$_2$ and systemic antibiotics were prescribed. Results were somewhat poor where resolution of the peri-implant disease was obtained in only 58% of the implants, seven implants were lost and four others continued to lose bone during the 5-year follow-up period.

Heitz-Mayfield et al (Heitz-Mayfield et al., 2012) evaluated access surgery and implant surface cleaning with titanium-coated curettes and by rubbing with gauze soaked in saline, plus prescription of systemic amoxicillin and metronidazole in 24 patients (36 implants) who presented with moderate to advanced peri-implantitis. At 12 months, all treated implants had PPD < 5 mm and 47% of the implants had no BoP. 92% of the patients had stable crestal bone levels or bone gain and only three implants in three patients had bone loss.

In summary, the optimum treatment modality for peri-implantitis remains unknown. Non-surgical therapy appears to be insufficient and surgical therapy is generally advocated. Adjunctive chlorhexidine application had only limited effects on clinical and microbiological parameters, whereas adjunctive local or systemic antibiotics were shown to reduce bleeding on probing and probing depths (Lindhe et al., 2008, Renvert et al., 2008).
1.4.4 Decontamination using air polishing devices

Whether or not a surgical or non-surgical, regenerative or resective approach is followed, removal of the biofilm and decontamination of the implant surface remains a necessity. The objective is to eliminate infection and resolve inflammation, rendering the surface conductive to bone regeneration and ideally re-osseointegration. Currently the use of abrasive air powder seems to provide sufficient implant detoxification and is a promising, economical and accessible method (Meyle, 2012), which will be discussed in detail in the following review.

1.4.4.1 Powders used in air polishing devices

1) Sodium bicarbonate

Sodium bicarbonate has generally been the most popular polishing powder used for this. In vitro studies indicate that they may also be potentially useful for the treatment of peri-implant infections. Using SEM, it was shown that the most efficient treatment that yielded a completely plaque-free surface on all implants regardless of their surface characteristics was sodium bicarbonate air-powder spray, whereas plastic curettes yielded only a partial removal of the biofilm layer since it was not possible to remove plaque from the depths of the implant threads, and the least effective was chlorhexidine 0.1% (Augthun et al., 1998). The same findings were confirmed in another study, in which 100% of bacteria were removed from implants with titanium plasma sprayed surfaces subjected to air-powder blasting with sodium carbonate (Parham et al., 1989). Moreover, 3 surfaces with different surface roughness (Ra = 0.17 µm, 1.14 µm and 3.17 µm) with an in vitro biofilm containing an average of 10³, 10⁴ and 10⁵ of S. sanguinis cells showed a completely cell free surface after being blasted for one minute with a sodium bicarbonate air abrasion device (Pereira da Silva et al., 2005). Similarly, implants coated with P. gingivalis endotoxin were more effectively cleaned with sodium bicarbonate air polishing compared to burnishing with distilled water, chlorhexidine or citric acid (Dennison et al., 1994).

Although it has been documented that sodium bicarbonate is highly abrasive to root cementum or dentin (Petersilka et al., 2003a) similar effects were not observed on titanium. The literature indicates that sodium bicarbonate air-polishing does not lead to significant alterations of the abutment (McCollum et al., 1992), the implant surface or its coating...
(Chairay et al., 1997, Augthun et al., 1998, Mengel et al., 1998). Only minor alterations were observed in surface topography of plasma sprayed implants that consisted of rounding of angles and edges of the plasma-spray coating and occasional surface pitting (Augthun et al., 1998, Parham et al., 1989). This was also the case with surface changes documented microscopically on sandblasted acid-etched surfaces, where sharped-edged elevations were markedly flattened and smoother after 40 seconds (Schwarz et al., 2009a) and 60 seconds of blasting (Kreisler et al., 2005). On machined implants however, Augthun et al. reported no surface alterations after 60 seconds treatment with sodium bicarbonate, whereas Duarte et al. (Duarte et al., 2009b) observed minute crater-like defects on smooth surfaces as a result of the powder crystals hitting the implant surfaces.

A clinical study has shown that surgical treatment of peri-implantitis which included mechanical removal of the biofilm using carbon curettes followed by sodium bicarbonate air abrasion was effective in reducing pocket depths by an average of 3 mm and reducing levels of *T. denticola*, *T. forsythia*, *P. micra*, and *F. nucleatum ss. nucleatum* when evaluated three months after treatment (Maximo et al., 2009).

Nevertheless, with a mean particle size of 75 µm and density of 2.16 g/cm³, the use of sodium bicarbonate subgingivally has not been recommended because of its abrasiveness and potential risk of soft tissue trauma (Kozlovsky et al., 2005).

### 2) Glycine Powder

Recently, glycine has been proposed as an alternative to the traditional sodium bicarbonate powder in order to enable the application of periodontal maintenance subgingivally without inducing substantial soft and hard tissue damage.

Glycine (greek = sweet) is a nonessential amino acid and an important component of most polypeptides that has very low toxicity, is non-allergenic and has a light, sweet taste and is thus commonly used in the food industry as a flavour enhancer. Glycine is odourless, colourless and is highly water-soluble making it suitable for intra-oral use. Previous studies have characterized these powder particles as having an average size of ~25 µm which is approximately four times smaller than that of conventional sodium bicarbonate powder (Schwarz et al., 2009a). This powder has a lower density (1.61 g/cm³) than sodium bicarbonate (Schwarz et al., 2009a) and is less hard, making it 80% less abrasive (Flemmig
et al., 2007). This was demonstrated on titanium implant surfaces, where it was shown that 40 seconds of treatment did not affect the surface topography under the scanning electronic microscope (Schwarz et al., 2009a). In a comparative study, 10 different instruments which were manual plastic curettes, manual carbon curettes, polishing cups, prophylaxis brushes, an ultrasonic scaler with a either a plastic tip or a carbon tip, an air scaler with a plastic tip, an ER: YAG laser, the vector system, and air-polishing with glycine were assessed for their effectiveness in the removing a 3-day in vitro S. mutans biofilm from 4 different titanium surfaces. Air polishing with glycine delivered the cleanest surfaces with the least surface alterations compared to all the other cleaning methods assessed (Schmage et al., 2012).

Schwarz et al. (Schwarz et al., 2009a) have demonstrated it’s efficiency in removal of biofilms from sandblasted acid etched titanium surfaces, by measuring the amount of residual stained biofilm, using a software programme to analyze digital images obtained using a light microscope and found that the biofilm remaining after blasting was within the range of 0.0% ± 0.0% to 5.7% ± 5.7%. According to these findings and compared to other studies by the same authors in which biofilm removal was assessed using the same splint system, air-powder systems were more efficient than irradiation with an Er:YAG (5.8% ± 5.1%), or an Er,Cr:YSGG laser (9.8% ± 6.2% to 53.8% ± 2.2%), the application of modified ultrasonic devices (28.3% ± 2.0% to 36.8% ± 4.5%), or plastic curettes in combination with chlorhexidine (58.5% ± 4.9% to 61.1% ± 11.4%) (Schwarz et al., 2006b, Schwarz et al., 2005a, Subramani and Wismeijer, 2012).

In a similar study, but using a clinically more comparable model, Sahrmann and co-workers (Sahrmann et al., 2013) investigated the efficiency of air polishing with glycine to remove biofilms from implants that were embedded in acrylic models that simulated different peri-implant defect morphologies. They used a permanent marker to resemble the presence of biofilm and the amount of residual biofilm after blasting was assessed using digital images and a specifically designed computer software. Comparisons were made between cleaning efficiency on coronally facing threads, apically facing threads and perpendicular to the implant surface and revealed that especially in narrow defects and in areas with compromised accessibility such as apically facing threads, it was not possible to completely remove the plaque biofilm, while cleaning efficiency was improved the wider the defect. Even though residual biofilm stains remained in all defect types, air blasting displayed a
reasonable technique for biofilm removal in areas that would be difficult to reach by conventional mechanical means (i.e. manual or ultrasound curettes). The cleaning observed on the apical threads was attributed to the ricochet effect, which produces a radial corona of secondary jets in addition to the central beam of the air-flow device, parallel to the surface to be treated and contributing to its efficiency.

A randomized control trial with a split mouth design that included 27 patients indicated that subgingival plaque removal using glycine powder in pockets of 3 – 5 mm resulted in a significantly greater reduction in sub-gingival bacterial counts and was more comfortable for patients than hand instrumentation (Petersilka et al., 2003b).

Another trial with 50 patients confirmed that air polishing with glycine was indeed more comfortable and quicker in subgingival biofilm removal than hand or ultrasonic scalers in periodontal pockets with probing depths (PDs) ≥ 5 mm, but a more pronounced reduction in total bacterial counts and specific bacterial strains was observed with conventional SRP using curettes compared to those with air-polishing and hand instrumentation was also superior in reducing inflammation as assessed by BOP 7 days after treatments. They concluded that air polishing might be useful for periodontal patients with residual pockets in the maintenance phase, provided that mineralized deposits are not present; otherwise conventional debridement techniques would be necessary beforehand for the removal of calculus (Moene et al., 2010).

The same findings were observed in a pilot clinical study on dental implants in which the efficiency of glycine powder air polishing as an adjunct in the treatment of peri-implant mucositis was evaluated. 12 patients were treated using an ultrasonic scaler with carbon fiber tips and in 12 patients adjunctive glycine blasting was used in pockets ≥ 4 mm. Bleeding index and pocket depth reduction were measured at 1 month and 3 months post-treatment. Results indicated that significant reductions were obtained by both methods, but mechanical debridement alone demonstrated greater bleeding reduction after 1 week. This may have been caused by the damage to the mucosa from high-pressure glycine slurry. Hence, adjunctive glycine polishing seemed to have a limited beneficial effect as compared with mechanical debridement alone, but in situations with deep pockets, narrow defects, or implant threads exposure where conventional mechanical debridement is hard to eliminate.
pathogenic biofilms, air abrasion with glycine might show some additional effects (Ji et al., 2013).

Contrary to these findings, a randomized controlled trial assessing the effectiveness of non-surgical management of moderate peri-implantitis found less bleeding on probing after 6 months with glycine air polishing compared to mechanical debridement + chlorhexidine (43.5 ± 27.7% versus 11.0 ± 15.7% reduction in BOP respectively). In this study, 15 patients were allocated to receive glycine air polishing and 15 patients were allocated to receive manual debridement with carbon curettes supplemented with chlorhexidine disinfection. Other clinical parameters that were assessed, which included pocket and clinical attachment levels showed limited improvements and were comparable for both groups (Sahm et al., 2011).

42 patients who presented with peri-implantitis were treated non-surgically with either air polishing using glycine (21 patients, 45 implants) or ER:YAG laser (21 patients, 51 implants). There were no differences in the results for both treatments after 6 months follow up. Both treatments showed limited improvement in pocket depths and bone loss, but there was a significant decrease in BOP and suppuration, but this could have been related to the significant improvement in oral hygiene observed (Renvert et al., 2011).

3) **Bioactive glass (BAG)**

BAG is another powder that could be potentially promising for the decontamination of implants with peri-implantitis. The original bioactive glass 45S5 composition is made up of 45% silica (SiO₂), 24.5% calcium oxide (CaO), 24.5% sodium oxide (Na₂O), and 6% phosphorous pentoxide (P₂O₅) in weight percentages. This powder has a mean particle size of 56 µm (Milly et al., 2014). When exposed to body fluids, a series of surface reactions occur leading to the development of a silica gel and a calcium phosphate layer on their surface. Successively, the calcium phosphate layer will recrystallize into hydroxycarbonate apatite, which is a bioactive apatite layer that gives BAG the capability of bonding with soft and hard tissues. They also possess osteoinductive properties through the release of ions that stimulate osteoprogenitors to differentiate into mature osteoblasts (Hench, 2006).

Their uses have mainly included bone repair and bone regeneration in maxillofacial and periodontal procedures. A clinical investigation carried out by Froum et al. (Froum et al.,
that evaluated BAG and compared it to open debridement in the treatment of 59 periodontal osseous defects in 16 patients, demonstrated that BAG significantly improved healing and resulted in significant improvement in osseous defect fill upon re-entry of the defect sites at 12 months. There was also greater probing depth reduction and greater gain in clinical attachment.

Bioactive glasses have expressed antimicrobial properties against oral microorganisms *in vitro* (Stoor et al., 1998). In addition, BAG abrasion has been proposed for the removal of carious dentine as research has shown it to be more selective than alumina, the most commonly used powder in minimally invasive dentistry (Paolinelis et al., 2008). Another possibility for intra-oral use that has been explored is the treatment of dentinal hypersensitivity where a clinical study has indicated that air-polishing using BAG was more effective in desensitizing previously sensitive teeth whilst providing better polishing characteristics and greater patient comfort compared to sodium bicarbonate. This was evaluated directly after the procedure and 10 days later (Banerjee et al., 2010).

Research on the incorporation of BAG on titanium has indicated increased bone to implant contact after blasting with a mixture of bioceramics in a rabbit model (Mueller et al., 2003). *In vitro*, titanium surfaces that were coated with BAG were effective in stimulating human osteoblast growth and differentiation, showing three times more cell proliferation than uncoated titanium alloy at 16 days. SEM observations of these BAG coated surfaces showed cells that were attached and completely spread (Oliva et al., 1998). Similarly another study using a human osteoblast-like cell line (MG63) revealed that Bioglass formed a suitable substrate for cell proliferation and function and was comparable to titanium alloy (Price et al., 1997). This indicated that BAG had an important potential as an implant coating.

1.4.4.2 Concerns on using air-polishing devices

In addition to surface alterations and soft tissue trauma, which have been discussed before, one of the major concerns of using a high air pressurized device intra-orally and especially subgingivally is tissue emphysema. However, only a few cases have been reported in the literature, one was in 1988 after routine periodontal maintenance around a molar with severe attachment loss that approached the root apices (Finlayson and Stevens, 1988),
another was during conservative caries removal using air abrasion (Liebenberg and Crawford, 1997) and a third case around implants during regular peri-implant prophylaxis (Bergendal et al., 1990). None of the 150 patients treated in the clinical studies described previously (125 with glycine and 25 with sodium bicarbonate) developed emphysema.

1.4.4.3 Biocompatibility of titanium surfaces decontaminated using air-polishing devices

Only a few in vitro studies have evaluated cell response and biocompatibility of titanium surfaces that have been treated with air-powder polishing devices, however only one has used glycine and none have used BAG (Table 5).

Shibli et al. (Shibli et al., 2003) investigated the effect of air-polishing treatment on the biocompatibility of sterile commercially pure titanium abutments (Sterngold, ImplaMed, Attleboro, MA, USA). In this study, the abutments were exposed for 30 seconds to sodium bicarbonate blasting and then fibroblasts were cultured on them for 24 hours. Cell numbers and morphology was assessed via SEM, which indicated lower cell numbers on air-blasted surfaces compared to control surfaces. However, no morphological alterations were observed on the surfaces.

The remaining four studies were conducted on implant surfaces that were previously contaminated with biofilms and all suggest the biocompatibility of titanium surface following air polishing to be high.

Parham et al. (Parham et al., 1989) did not find any differences in the number of attached fibroblasts between plasma-sprayed titanium surfaces subjected to air-powder sodium bicarbonate treatment and control surfaces.

Similar results were observed in another study in which subjects wore stents for 7 days to grow biofilms on dental implants. The implants were cleaned using plastic scalers or an air-powder system using sodium bicarbonate. Fibroblasts were cultivated for 24 hours in vitro and cell vitality was assessed using vital staining whereas cell spreading was evaluated using SEM. Results showed that implants that were cleaned with the air-powder system showed a similar percentage of vital cells compared to the control group of non-contaminated sterile implants. In contrast, implants treated with plastic scalers showed a
50% reduction in cell numbers and reduced cell spreading. This difference was attributed to the differences in cleaning efficiency in favour of the air-powder system (Augthun et al., 1998).

Schwarz et al. (Schwarz et al., 2009a) evaluated the influence of different air-abrasive powders on cell viability at biologically contaminated dental implant surfaces. In this study, intraoral splints were worn to grow an in vivo biofilm on SLA titanium discs (Institut Straumann AG, Basel, Switzerland) for 48 h, and were then decontaminated by air polishing using different commercial powders, 3 of which were powders containing glycine and a fourth containing sodium bicarbonate. Discs were autoclaved, and then SaOs-2 cells were cultured for 7 days. Cell viability was quantified using mitochondrial activity and was significantly greater on surfaces treated with sodium bicarbonate compared to those treated with glycine, however both showed significantly lower cell viability than control sterile non-contaminated surfaces. The authors suggested that sodium bicarbonate particles being harder and more abrasive induced a minor degree of surface ablation that could have been necessary to improve cell viability at biologically contaminated titanium implants.

To compare between ER: YAG laser and air-powder polishing, Kreisler et al. (Kreisler et al., 2005) incubated SLA titanium discs (Friadent GmbH, Germany) with P. gingivalis for one hour to form a biofilm, and then discs were subjected to either ER:YAG laser or air-polishing for 60 seconds, but they did not mention which powder was used. Fibroblast cell proliferation was assessed using a colourimetric assay to assess metabolic cell activity at 12, 24, 48 and 72 hours. Comparisons were also made with cell proliferation on contaminated and untreated discs in addition to sterile discs that acted as controls. There was no difference in the proliferation rates on both air-powder and Er:YAG laser treated surfaces and both were comparable to the controls. On the other hand, a significantly lower proliferation rate was observed on the contaminated, non-treated specimens compared to all other groups.
<table>
<thead>
<tr>
<th>Study</th>
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<td>(Shibli et al., 2003)</td>
<td>CP Ti</td>
<td>No biofilm</td>
<td>Sodium bicarbonate</td>
<td>SEM – fibroblasts</td>
<td>Lower cell numbers on air-blasted surfaces compared to controls</td>
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<td>(Parham et al., 1989)</td>
<td>TPS</td>
<td><em>in vivo</em></td>
<td>Sodium bicarbonate</td>
<td>SEM - fibroblasts</td>
<td>No difference in cell numbers between air-blasted surfaces and controls</td>
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| (Augthun et al., 1998)       | TPS, HA machined | *in vivo*  | Sodium bicarbonate       | Vital staining/ SEM fibroblasts | Similar percentage of vital cells to control group.  
Plastic scalers showed 50% decrease in cell numbers |
| (Kreisler et al., 2005)      | SLA      | *P. gingivalis* | Air-powder (does not mention type) ER:YAG laser | alamarBlue/ Fibroblasts     | No difference in cell proliferation rates                                      |
| (Schwarz et al., 2009a)      | SLA      | *in vivo*     | Glycine                  | ATP-based luminescent cell viability assay | Controls > sodium bicarbonate > glycine                                      |

*Table 5 In vitro studies evaluating cell response on air-powder treated titanium implant surface*
CHAPTER 2 THE MICROBIOTA AROUND IMPLANTS ASSOCIATED WITH PERI-IMPLANTITIS AND HEALTH (STUDY 1)
2.1 INTRODUCTION

The general agreement from early studies seems to be that healthy implants are surrounded by a microflora similar to that found around periodontally-healthy teeth, consisting primarily of cocci and non-motile rods, a low number of Gram-negative anaerobic species and low detection levels of “periodontal pathogens” and spirochaetes (Adell et al., 1986, Lekholm et al., 1986a, Bower et al., 1989, Ong et al., 1992, George et al., 1994, Mombelli et al., 1987, Quirynen and Listgarten, 1990). On the other hand, failing implants have been characterized by a significantly higher proportion of Gram-negative anaerobic rods, spirochaetes and showed a decrease in coccoid cells (Alcoforado et al., 1991, Leonhardt et al., 1999, Mombelli et al., 1987, Listgarten and Lai, 1999, Rams et al., 1984).

Species that appeared to consistently predominate around healthy implants included streptococci, Peptostreptococcus, Fusobacteria, Actinomyces and Veillonella species (Lekholm et al., 1986b, Mombelli et al., 1987, Maxson et al., 1992). A wide range of species has been implicated in implant failure. *Fusobacterium* species, *P. intermedia*, *Wolinella recta* (now *Campylobacter rectus*) and *Capnocytophaga* were some of the earliest species that were described in relation to failing implants (Mombelli et al., 1987, Rosenberg et al., 1991, Listgarten and Lai, 1999).

A few studies reported that *A. actinomycetemcomitans*, staphylococci and yeasts were also detected from around failing implants (Alcoforado et al., 1991, Rosenberg et al., 1991, Rams et al., 1990), possibly implicating these infrequently observed microorganisms in the aetiology of peri-implantitis. However, in most other studies these organisms, especially *A. actinomycetemcomitans* and yeasts could not be detected (Tabanella et al., 2009, Papaioannou et al., 1996, Listgarten and Lai, 1999).

Organisms such as *P. gingivalis* and *C. rectus* were rarely detected in earlier studies (Mombelli and Mericske-Stern, 1990). This was explained by the fact that most examined patients with implants at that time were completely edentulous and it has been suggested that patients have significantly fewer ‘periodontopathogens’ when rendered edentulous. (Danser et al., 1994), however recent studies have all found these organisms in fully edentulous patients (Devides and Franco, 2006, Quirynen and Van Assche,
proving that it was not true that the microbial load ‘disappears’ following tooth extraction.

It has been accepted that the bacteria associated with implants are related to those already resident in the oral cavity, that is the remaining teeth can act as reservoirs for the colonization of the subgingival environment around implants in partially edentulous patients (Apse et al., 1989, Quirynen and Listgarten, 1990, Quirynen et al., 1996b, Papaioannou et al., 1996, Takanashi et al., 2004, Sumida et al., 2002). The exact mechanisms by which these peri-implant pockets are colonized are still unclear. Recently, the theory of ‘microbial translocation’ has been debated. Evidence has emerged to show that there is a significant partitioning between both periodontal and peri-implant microbial profiles (Koyanagi et al., 2013, Kumar et al., 2012). Peri-implantitis appears to be a site-specific infection. This has been supported in many studies where healthy implants had a similar microbial profile regardless of whether or not the patients had other implants exhibiting peri-implantitis (da Silva et al., 2013, Mombelli et al., 1987, Sanz et al., 1990, Hultin et al., 2002).

After surgical exposure of implants during placement of abutments, rapid colonization of the surfaces occurs (van Winkelhoff et al., 2000). It then takes between 13 – 26 weeks for a stable subgingival microbiota to form around the implants (Quirynen et al., 2006). Some studies using experimentally induced gingivitis/periodontitis models have demonstrated that the progression of disease around implants is accompanied by a similar shift in microbiota to that which occurs around teeth. This being highlighted by the increase in Gram-negative and strictly anaerobic bacteria (Leonhardt et al., 1992).

Most of our knowledge regarding the microbial flora surrounding implants have come from studies that utilized culture techniques and/ or direct phase contrast microscopy. More recently, molecular techniques such as DNA/DNA hybridization, using whole-genomic and oligonucleotide probes have shown that other bacteria are also found in significantly greater numbers in failing implants, namely: *P. gingivalis, T. forsythia, T. denticola, F. nucleatum ss. nucleatum, F. nucleatum ss. vincentii, E. corrodens, Campylobacter sp. and P. micra* (Shibli et al., 2008, Papaioannou et al., 1996, Tabanella et al., 2009). However most studies have found that the bacteria associated with peri-implantitis were also found in healthy gingival crevices without any signs of destructive processes (Leonhardt et al., 1993, Quirynen et al., 2006, Shibli et al., 2008, Renvert et al., 2007, Meijndert et al., 2010), that their presence could not be used as a
marker to predict future disease (Meijndert et al., 2010) and that there were no significant correlations between the presence of these bacteria and the histological degree of inflammation (Ferreira et al., 2009).

In conclusion, these findings indicate that the microbial community in peri-implantitis is complex. The great variability in study designs, methods used and presentation of data might have contributed to some of the conflicting results. Although these studies have helped detect the presence of specific target bacteria, they do not describe the true diversity and complexity of the peri-implant community.

Thus the first part of this research was dedicated to identifying the microbial community around implants with and without peri-implantitis using an open-ended approach, which does not target specific bacteria.
2.2 Study 1 Aims and Objectives

Aim

The aim of this study was to identify and compare the microbiota around dental implants with and without a clinical and radiological diagnosis of peri-implantitis.

Objectives

1. To examine the microbial diversity in both clinical presentations.

2. To identify the predominant microorganisms around healthy implants and those associated with peri-implantitis.
2.3 Materials and Methods

Ethical approval for the study was obtained from the research ethics committee at King’s College Hospital, National Research Ethics Service (LREC Reference Number 09/ H0808/ 112)

2.3.1 Subject Recruitment

Twenty-one subjects presenting with implants in function for at least one year were enrolled in this study. The subjects were patients attending Guy’s Hospital either for implant maintenance after placement or for treatment of peri-implantitis. Sample collection started in March 2010 and was completed by end of July 2012. All subjects were partially edentulous and had had fixed restorations, ranging from single crowns to full arch screw-retained restorations. The subjects were assigned to two groups: a peri-implantitis group consisting of 11 patients who had at least one implant showing signs of peri-implantitis and a control group which consisted of 10 patients where all implants were healthy. This number of subjects was chosen according to a few factors. First, the literature was checked to compare how many numbers were reported in other studies. Then, preliminary statistical analysis was carried out after 13 patients, results of which are discussed in Appendix 2. Finally, recommendations which point out that studies with less than 20 patients are of negligible clinical and scientific value were taken into consideration. This was to ensure that the study had adequate statistical power to detect a clinically relevant risk and to correctly interpret negative results (Needleman et al., 2012).

Peri-implantitis was characterized by osseous defects of 3 mm or more from platform level (Figure 3), PPDs of 5 mm or more and an inflamed peri-implant mucosa exhibiting bleeding on probing and/or suppuration (Table 6). The study protocol was explained to each patient and signed informed consent was obtained.

The definition of peri-implantitis used in the present study followed the outline presented by the sixth consensus report of the EWOP (Lindhe et al., 2008). The other reference that was used to classify peri-implantitis in this study was the health scale (Table 7) for dental implants, which was devised during the consensus conference of the International Congress of Oral Implantologists (Misch et al., 2008). According to this
health scale, the definition of peri-implantitis used would fall into groups II (satisfactory survival) and III (compromised survival/ failing).

Figure 3 Mandibular implants showing saucer-shaped osseous defects characteristic of the bone loss around implants (radiograph of patient number 1)

<table>
<thead>
<tr>
<th>Peri-implantitis</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflamed peri-implant mucosa</td>
<td>Healthy peri-implant mucosa</td>
</tr>
<tr>
<td>Bone Loss ≥ 3mm</td>
<td>Bone Loss &lt; 1mm</td>
</tr>
<tr>
<td>Pocket Depth ≥ 5mm</td>
<td>Pocket Depth &lt; 4mm</td>
</tr>
<tr>
<td>BOP +/- suppuration</td>
<td>+/- BOP</td>
</tr>
</tbody>
</table>

Table 6 Inclusion criteria for patients enrolled in the study (BOP, bleeding on probing)
<table>
<thead>
<tr>
<th>Implant Quality Scale Group</th>
<th>Clinical Conditions</th>
</tr>
</thead>
</table>
| I. Success (optimum health) | a) No pain or tenderness upon function  
b) No mobility  
c) <2 mm radiographic bone loss from initial surgery  
d) No history of exudate |
| II. Satisfactory survival   | a) No pain on function  
b) 0 mobility  
c) 2–4 mm radiographic bone loss  
d) No history of exudate |
| III. Compromised survival  | a) May have sensitivity on function  
b) No mobility  
c) Radiographic bone loss > 4 mm (less than 1/2 of implant body)  
d) Probing depth >7 mm  
e) May have history of exudate |
| IV. Failure (clinical or absolute failure) | Any of following:  
a) Pain on function  
b) Mobility  
c) Radiographic bone loss >1/2 length of implant  
d) Uncontrolled exudate  
e) No longer in mouth |

*Table 7 Health Scale for Dental Implants, International Congress of Oral Implantologists. Pisa, Italy, Consensus Conference 2007 (Misch et al., 2008)*

**2.3.2 Exclusion Criteria**

Patients were excluded if they had taken antibiotics or anti-inflammatory drugs within the previous month, had received oral prophylactic procedures (eg. scaling) within the past 3 months or had a chronic medical disease or condition that might affect the oral flora e.g. diabetes mellitus, oral cancer, HIV infection and those using immunosuppressant medications, bisphophonates or steroids. Also subjects who were restored with removable prosthesis, i.e. overdentures and those who smoked more than 10 cigarettes per day were excluded (Table 6).
2.3.3 Clinical Assessment

2.3.3.1 Implant Selection

In each subject, the implant with the greatest pocket depth was selected for sampling. If two or more sites presented with the same pocket depth, the most accessible, usually the most anterior, implant was chosen.

2.3.3.2 Clinical and Radiographic Examination

Before sample collection, clinical data such as general oral hygiene, presence or absence of bleeding on probing around implants and probing pocket depths at 4 sites (midfacial, midlingual, mesial and distal) were recorded. If implants showed clinical signs of peri-implantitis, the level of bone loss was recorded on the mesial and distal surface of the implant using the most recent radiograph in the patient’s records. Bone level loss (average of mesial and distal measurements) was measured in millimetres between the implant shoulder and the first bone-to-implant contact. If measurements were taken from a DPT, a magnification of 1.25x was taken into consideration while digital radiographs were calibrated. It must be noted that it was sufficient to determine that the bone loss was more than 3mm rather than to accurately measure it. Other data were also collected such as medical health, reason for tooth loss, date implants were placed and loaded, date peri-implantitis was recorded, smoking status, parafunctional habits and the type and dimensions of the implants used. The form used to collect the clinical and radiographic data is shown in Appendix 3.

2.3.3.3 Sample Collection

Sampling sites were isolated with cotton rolls and the supragingival plaque was removed with cotton pellets. Subgingival plaque samples were obtained from the deepest parts of the buccal and lingual gingival sulci using a double-ended sterile curette (Dentsply, UK). One end was used for the buccal sample and the other for the lingual, and then both were pooled in a 7 ml polystyrene bijou bottle (Sterlin, UK) containing 1.0 ml of pre-reduced Fastidious Anaerobic Broth (FAB) by vigorously agitating the tip of the curette in the solution.
2.3.4 Microbiological Assessment

2.3.4.1 Processing of the Plaque Samples

Six sterile glass beads, 2 mm in diameter were added to the transport media containing the plaque samples and the samples were dispersed by vortexing for 10 seconds on a vortex mixer (Stuart auto vortex mixer, UK). They were then serially diluted from 10\(^{-1}\) to 10\(^{-5}\) and 0.1 ml aliquots of the ten-fold serial dilutions were plated in duplicates on non-selective agar plates: Fastidious Anaerobic Agar supplemented with 5% v/v defibrinated horse blood (FAA). The plates were incubated for 5 days at 37\(^{\circ}\)C in an aerobic incubator (LEEC Compact Incubator K2, LEEC, UK) and for 10 days at 37\(^{\circ}\)C in an anaerobic workstation (MACS-MG-1000-anaerobic workstation, Don Whitley Scientific, UK) in an atmosphere of 80% N\(_2\), 10% CO\(_2\), and 10% H\(_2\).

Media used for serial dilutions and plates designated for anaerobic incubation were all pre-reduced in the anaerobic workstation for 3 hours.

2.3.4.2 Calculation of Colony Forming Units (CFUs) and Subculturing of Colonies

After incubation, plates with between 30 and 300 colonies were counted and total aerobic and anaerobic viable counts expressed as colony forming units (CFU) / sample. 96 colonies (48 from the aerobically-incubated plates and 48 from the anaerobic plates) were then subcultured at random from the plates and incubated for a further 2 days. In addition, the colonies were stored in Brain Heart Infusion (BHI) broth supplemented with 10% glycerol (weight per volume) at -80\(^{\circ}\)C.

2.3.4.3 Identification of Bacteria using 16S Ribosomal (rRNA) Gene Sequencing

a) DNA Extraction

Colonies were suspended in 50 µl of sterile deionized water in 1.5 ml Eppendorf tubes, then vortexed and bacterial DNA was extracted by boiling for 10 minutes at 95\(^{\circ}\)C (Microtherm microtube incubator; Camlab, UK) with shaking at 50 rpm to disrupt the cells. After that they were cooled on ice for 1 minute, and then centrifuged for another minute at 13,000 rpm (Microlite – Thermoscientific, UK). The supernatant DNA was then aspirated to be used as a template for the PCR reaction.
b) PCR amplification

The PCR reactions were performed in sterile 0.2 ml microcentrifuge tubes in a total volume of 12.5 µl. The PCR reaction mixture contained 0.5 µl of extracted DNA, 0.25 µl of each primer (from a stock of 10 pmol/µl) and 11.5 µl of Reddymix (a ready to use master mix which contains MgCl₂, dNTP (0.2 mM of each), (NH₄)₂SO₄, Tris-HCL, Tween® 20, ThermoPrime Taq DNA Polymerase, a precipitant and a red dye for electrophoresis).

PCR amplification of a partial fragment of the 16S rRNA gene was performed using a Techne TC – 412 Thermal Cycler (Barloworld Scientific, Stone, Staffordshire, UK). The reaction sequence consisted of 34 amplification cycles, each involving dissociation of DNA at 94°C for 30 seconds, annealing of primers at 49°C for 30 seconds and primer elongation at 72°C for 90 seconds. Initial dissociation of DNA was for 10 minutes at 94°C and the final extension was for 5 minutes at 72°C.

The primers used for PCR amplification were the universal primers 9F and 907R (Table 8).

PCR products were then analyzed by electrophoresis on agarose gels (Sigma) with a GelRed™ nucleic acid gel stain (Cambridge Biosciences) in 0.5 x TBE Buffer for 10 – 12 minutes at 100V and visualized with UV light (Alpha Innotech Fluorchem Light Cabinet).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>9F</td>
<td>5’- GAGTTTGATCCTGGCTCAG-3’</td>
</tr>
<tr>
<td>907R</td>
<td>5’- CCGTCAATTCTTTAGTTT-3’</td>
</tr>
</tbody>
</table>

Table 8 Primer Sequences used for PCR

c) Purification and Cleaning of PCR products

Purification of the PCR products in order to eliminate unincorporated primers and dNTPs (deoxynucleotides) was carried out using microCLEAN (Microzone Ltd, UK) for the first 7 samples and ExoSAP-IT® (USB, Cleveland, OH, USA) for the rest of the
samples. For the former, equal volumes of microCLEAN and the PCR product were added together. They were mixed by pipetting and the mixture was left at room temperature for 5 minutes then centrifuged at 13000 rpm for 10 minutes. The supernatant was carefully removed and the tubes were centrifuged again for a further 10 seconds at 13000 rpm. Any remaining supernatant was then aspirated and discarded and the tubes were placed with lids open for 10 minutes in a vacuum to dry. An appropriate amount of sterile distilled water was subsequently added to rehydrate the pellet, the amount of which was determined according to the intensity of the band visualized by UV light in the previous step. The rest of the samples were cleaned and purified using ExoSAP-IT®. This was a simpler and faster method that gave more consistent and reliable results. It consisted of adding 2µl of ExoSAP-IT® to 5µl of the PCR product for a combined 7µl reaction volume. The mixture was incubated at 37°C for 15 minutes to degrade the remaining primers and nucleotides, followed by incubation at 80°C for a further 15 minutes to inactivate the ExoSAP-IT®.

**d) Sequencing of PCR products**

The PCR product was then sequenced immediately or stored at -20°C till the next day. Sequencing was accomplished using the 9F primer (except for the first sample which was also sequenced in both directions using 27F and 1492R), a Big Dye® Terminator Cycle Sequencing kit (Applied Biosystems, UK), and a 3730xl ABI Genetic Analyzer (Applied Biosystems, UK).

For each Big Dye Sequencing reaction a mixture of 4 µl of 1:15 dilution of the 9F primer (stock is 10 pmol/ µl concentration), 1.75 µl of sterile distilled water, 1.75 µl sequencing buffer and 0.5 µl Big Dye (Applied Biosystems) was added to 2.5 µl of the cleaned PCR product in a 96-well microtitre plate. After a brief spin at 400 g, the plate was placed in a Techne TC – 412 Thermal Cycler (Barloworld Scientific, Stone, Staffordshire, UK) and the PCR products were subjected to a cycle of denaturation (96 °C for 10 seconds), annealing (50 °C for 5 seconds) and extension (60 °C for 2 minutes). This cycle was repeated 30 times.

The products were then purified using the Big Dye Terminator clean up method. Ten µl of sterile distilled water was added to each well followed by 50 µl of a precipitation mixture (made up of 47 µl of 96% ethanol, 2 µl of a 3 M sodium acetate solution and 1 µl of 0.1 mM EDTA (Ethylenediaminetetraacetic acid)). This was incubated at room
temperature for 15 minutes and then centrifuged at 2750 g for 20 minutes at 4 °C (Eppendorf Centrifuge 5810 R). The supernatant was discarded by tipping the plate onto clean tissue and then by centrifuging the plate upside down on fresh tissue for 2 seconds at 400 g. Next, 100 µl of 70 % ethanol was added to the wells and the plate was centrifuged at 2750 g at 4 °C for 5 minutes. The supernatant was discarded as described before and the plate was placed in a vacuum unsealed for 10 minutes. Finally, 10 µl of 1/10 TE buffer was added to each well to rehydrate the DNA and the DNA sequences were determined using the 3730xl DNA analyzer (Applied Biosystems, UK).

2.3.5 Data Analysis

Most sequences were matched against sequences of reference organisms using the Human Oral Microbiome Database (HOMD) extended V1.1 and version 13.2 (Chen et al., 2010). In some instances, it was necessary to also compare the sequences to those stored in the NCBI sequence database using the BLAST (Basic Local Alignment Search Tool) algorithm (Johnson et al., 2008) or to use the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Only sequences that were longer than 350 base pairs and had an identity match of 98.5% or greater were considered in the results. The software BioEdit 7.2.5 (Hall, 1999) was used to edit and analyze sequences. Sequences were aligned with the ClustalW multiple alignment application (Thompson et al., 1994) accessed through BioEdit and were corrected by manual inspection. Phylogenetic trees were constructed using the MEGA 5.2 software (Tamura et al., 2013a) and the Interactive Tree of Life website (http://itol.embl.de/index.shtml) (Letunic and Bork, 2011)

The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Jukes-Cantor method and a 500 replicate bootstrap analysis. A bootstrap consensus tree was calculated and average bootstrap values were included on the trees (Saitou and Nei, 1987, Felsenstein, 1985)

2.3.6 Statistical Analyses

Demographic variables and clinical parameters were compared for significant differences between groups using the Mann-Whitney U-test, independent samples t-test or Fisher’s exact test according to whether or not the data was normally distributed.
As the variance was very large for the data obtained from the quantitative culture counts (after aerobic and anaerobic incubation) and did not follow normal distribution, a log transformation was used. This normalized the data and an independent samples t-test was used to compare the means of this transformed variable. The aerobic and anaerobic data were analyzed separately to avoid double counting the facultative organisms that will grow in both atmospheres.

The relative abundance of species in each group (peri-implantitis and healthy) was calculated based on the presence or absence of each species i.e. species were counted as present/absent irrespective of the number of isolates and then divided by the number of samples in each group. The distribution of species in peri-implantitis and healthy implants was compared using Fisher’s exact test.

For descriptive statistics (percentages of isolates), the data was normalized to account for differences in group sizes. Samples were corrected to 84 species each and then proportions of species, genera and phyla were calculated.

Statistical analyses were performed using the data-processing software SPSS (Version 21 for Mac; SPSS, Chicago, IL). The level of significance was set at p < 0.05.

2.3.7 Phenotypic Differentiation of Streptococcus and Actinomyces

Although 16S rRNA sequence homology analysis is a useful tool for bacterial phylogenetic study, it cannot provide sufficient resolution to differentiate between some closely related species (Janda and Abbott, 2007).

Isolates provisionally identified as Streptococcus constellatus or intermedius were further characterized by means of the sialidase test. Fifty µl of a bacterial suspension in TES buffer (n-Tris hydroxymethyl 2-aminoethanesulphonic acid buffer, pH7.5) (Sigma) was added to 20 µl of a fluorescent-labeled substrate (2’-4-methylumbelliferyl α-D-N-acetylneuraminic acid at a working concentration of 100 µg/ml) (Sigma) in a 96-well flat-bottomed microtitre tray. The trays were covered and incubated aerobically at 37°C for 3 hours. Substrate hydrolysis was observed by measuring fluorescence at excitation and emission wavelengths of 355 and 460 nm respectively using a fluorimeter with a plate reader attachment (Fluoroskan plate reader, Labsystems iEMS Reader MF). S. intermedius is known to be positive in this test, whilst the test is negative for S.
constellatus (Whiley et al., 1990). P. gingivalis was used as the positive control for the sialidase test (Moncla et al., 1990).

To differentiate between A. oris and A. naeslundii, partial sequencing based on the met-G (methionyl-tRNA synthase, ANA_1898) house-keeping gene was used (Henssge et al., 2009). After DNA extraction using the method described above, gene amplification was carried out using a set of multiple met-G primers (Table 9). A similar PCR programme to the one described for 16S rRNA was used, the only difference being the primer annealing temperature, which was 55 °C instead of 49 °C.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
<th>Sequencing primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MetG-F</td>
<td>5’-GCCGACCAGCAACAAC- 3’</td>
<td>5’-GAGGTCGTCTCCAGCG</td>
</tr>
<tr>
<td></td>
<td>5’-GCCTGTCTACGACCTGTC- 3’</td>
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</tr>
<tr>
<td></td>
<td>5’-ACTTCATGGGAACGAA- 3’</td>
<td></td>
</tr>
<tr>
<td>MetG-R</td>
<td>5’-CGGAGTCGGCCTAACAG- 3’</td>
<td>5’-CAGGAAGGCGAGCAC-GAT- 3’</td>
</tr>
<tr>
<td></td>
<td>5’-ACTCGTCCAGCTGGTGA- 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’-CGGTGATGACGCGGTAGC- 3’</td>
<td></td>
</tr>
</tbody>
</table>

Table 9 Met-G primers; For each PCR reaction, 0.2µl of each primer was used with 1µl of the DNA extraction (a total of 15µl for each PCR reaction).

Finally, the PCR products were cleaned using ExoSAP-IT® and DNA fragments were sequenced using both forward and reverse primers. Sequence analysis was completed in the same manner as previously described for 16S rRNA, but strains were identified by BLAST searching using the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
2.4 Results

2.4.1 Descriptive and Clinical Data for Patients Included in the Study

A total of 21 subjects (10 with healthy implants and 11 with peri-implantitis) were enrolled in this study. There were 14 women and 7 men with an age range from 20 – 82 years (mean age 55 years). Demographical data for the patients are presented in Table 10 and the clinical parameters for the implant sites in Table 11.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Smoker</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (p)</td>
<td>63</td>
<td>F</td>
<td>No</td>
</tr>
<tr>
<td>2 (h)</td>
<td>20</td>
<td>M</td>
<td>No</td>
</tr>
<tr>
<td>3 (h)</td>
<td>38</td>
<td>M</td>
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</tr>
<tr>
<td>4 (p)</td>
<td>64</td>
<td>F</td>
<td>No</td>
</tr>
<tr>
<td>5 (h)</td>
<td>82</td>
<td>F</td>
<td>No</td>
</tr>
<tr>
<td>6 (p)</td>
<td>52</td>
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<td>8 (p)</td>
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</tr>
<tr>
<td>9 (h)</td>
<td>78</td>
<td>F</td>
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</tr>
<tr>
<td>10 (h)</td>
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<td>F</td>
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</tr>
<tr>
<td>11 (p)</td>
<td>45</td>
<td>M</td>
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</tr>
<tr>
<td>12 (p)</td>
<td>55</td>
<td>M</td>
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</tr>
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<td>F</td>
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</tr>
<tr>
<td>15 (h)</td>
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</tr>
<tr>
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<tr>
<td>17 (p)</td>
<td>60</td>
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</tr>
<tr>
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<td>35</td>
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</tr>
<tr>
<td>21 (p)</td>
<td>61</td>
<td>F</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 10 Descriptive data for patients included in the study. (h), healthy implant (p), peri-implantitis
<table>
<thead>
<tr>
<th>Subject</th>
<th>Pdl status</th>
<th>Implant Type</th>
<th>Implant Surface</th>
<th>Implant location</th>
<th>Screw/cement</th>
<th>Implant length (mm)</th>
<th>Years in function*</th>
<th>Prosthetic loading (year)</th>
<th>Pi recorded (year)</th>
<th>Average PPD (mm)</th>
<th>Bone loss (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (p)</td>
<td>P</td>
<td>Astra</td>
<td>Osseospeed</td>
<td>LR3 screw</td>
<td>13</td>
<td>7</td>
<td>2005</td>
<td>2005</td>
<td></td>
<td>9</td>
<td>6</td>
</tr>
<tr>
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<td>Astra</td>
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<td>4</td>
<td>2008</td>
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<td>0</td>
</tr>
<tr>
<td>3 (h)</td>
<td>H</td>
<td>Brånemark</td>
<td>Machined</td>
<td>UR3 screw</td>
<td>15</td>
<td>14</td>
<td>1998</td>
<td>-</td>
<td>2</td>
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</tr>
<tr>
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<td>Machined</td>
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<td>18</td>
<td>1994</td>
<td>1997</td>
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<td>Machined</td>
<td>LR3 screw</td>
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<td>21</td>
<td>1991</td>
<td>-</td>
<td>3</td>
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<td></td>
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<td>H</td>
<td>Astra</td>
<td>TiOblast</td>
<td>UR5 screw</td>
<td>11</td>
<td>12</td>
<td>1998</td>
<td>2002</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>7 (h)</td>
<td>G</td>
<td>Astra</td>
<td>Osseospeed</td>
<td>UR2 N/A</td>
<td>15</td>
<td>2</td>
<td>2009</td>
<td>-</td>
<td>4</td>
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<td>Osseospeed</td>
<td>LR5 screw</td>
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<td>2006</td>
<td>2011</td>
<td>7</td>
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<td>H</td>
<td>Astra</td>
<td>Osseospeed</td>
<td>UR4 screw</td>
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<td>6</td>
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<td>-</td>
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</tr>
<tr>
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<td>H</td>
<td>Brånemark</td>
<td>Machined</td>
<td>UL1 screw</td>
<td>15</td>
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<td>1993</td>
<td>-</td>
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<td></td>
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<td>G</td>
<td>Astra</td>
<td>TiOblast</td>
<td>UL3 cement</td>
<td>13</td>
<td>8</td>
<td>2004</td>
<td>2010</td>
<td>4.75</td>
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<tr>
<td>12 (p)</td>
<td>G</td>
<td>Astra</td>
<td>Osseospeed</td>
<td>UL2 screw</td>
<td>11</td>
<td>2</td>
<td>2010</td>
<td>2010</td>
<td>7.5</td>
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<tr>
<td>13 (p)</td>
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<td>Astra</td>
<td>Osseospeed</td>
<td>LL1 cement</td>
<td>11</td>
<td>4</td>
<td>2008</td>
<td>2011</td>
<td>5</td>
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<tr>
<td>14 (p)</td>
<td>G</td>
<td>Astra</td>
<td>Osseospeed</td>
<td>UR2 screw</td>
<td>13</td>
<td>5</td>
<td>2007</td>
<td>2007</td>
<td>8.3</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Table 11 Clinical parameters for implant sites. Pi, peri-implantitis; PPD, probing pocket depth; (p), peri-implantitis; (h), healthy implants; Pdl, periodontal; H, Healthy. G, gingivitis. P, periodontitis
<table>
<thead>
<tr>
<th>Time</th>
<th>Manufacturer</th>
<th>Model</th>
<th>Material</th>
<th>Screw Type</th>
<th>Length</th>
<th>Diameter</th>
<th>Production Year</th>
<th>Post-Production Year</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 (h)</td>
<td>G Brånemark</td>
<td>Machined</td>
<td>UR2</td>
<td>screw</td>
<td>13</td>
<td>18</td>
<td>1994</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>16 (h)</td>
<td>H Brånemark</td>
<td>Machined</td>
<td>UR1</td>
<td>screw</td>
<td>13</td>
<td>15</td>
<td>1997</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>17 (p)</td>
<td>G Astra</td>
<td>Osseospeed</td>
<td>UL5</td>
<td>screw</td>
<td>11</td>
<td>6</td>
<td>2012</td>
<td>2012</td>
<td>7</td>
</tr>
<tr>
<td>18 (h)</td>
<td>P Brånemark</td>
<td>Machined</td>
<td>UR4</td>
<td>screw</td>
<td>15</td>
<td>18</td>
<td>1994</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>19 (h)</td>
<td>G Astra</td>
<td>Osseospeed</td>
<td>UR1</td>
<td>cement</td>
<td>15</td>
<td>5</td>
<td>2007</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>20 (p)</td>
<td>G Astra</td>
<td>Osseospeed</td>
<td>LL2</td>
<td>screw</td>
<td>15</td>
<td>5</td>
<td>2007</td>
<td>N/A</td>
<td>7</td>
</tr>
<tr>
<td>21 (p)</td>
<td>G Astra</td>
<td>TiOblast</td>
<td>UL 4</td>
<td>screw</td>
<td>13</td>
<td>8</td>
<td>2004</td>
<td>2012</td>
<td>8</td>
</tr>
</tbody>
</table>
Table 12 provides an overall summary of patient and clinical data. It can be seen that the mean age was comparable between groups. As expected, mean bone loss and pocket depths were significantly greater ($p<0.001$) in peri-implantitis sites (5.8 mm and 7 mm respectively) than in healthy ones (0.3 mm and 2.9 mm respectively). Examples of radiographs from sites sampled in the study can be seen in Figure 4 where one can appreciate the extent of bone loss around failing implants. The implants had been in function ranging from 2 – 21 years (average of 9.7 years).

<table>
<thead>
<tr>
<th></th>
<th>Healthy Implants (n=10)</th>
<th>Peri-implantitis (n=11)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>55.4 ± 18.9</td>
<td>54 ± 11.8</td>
<td>0.84 ($t$-test)</td>
</tr>
<tr>
<td><strong>Gender</strong> (male:female)</td>
<td>3 : 7</td>
<td>5 : 6</td>
<td>0.66 (FE)</td>
</tr>
<tr>
<td><strong>Implant type</strong></td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Astra</td>
<td>4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Brånemark</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Years in function</strong></td>
<td>12.3 ± 7</td>
<td>7.4 ± 4.4</td>
<td>0.065 ($t$-test)</td>
</tr>
<tr>
<td><strong>PPD (mm)</strong></td>
<td>2.9 ± 1.1</td>
<td>7 ± 1.4</td>
<td>$&lt;0.001**$ ($t$)</td>
</tr>
<tr>
<td><strong>Radiographic bone loss (mm)</strong></td>
<td>0.3 ± 0.5</td>
<td>5.8 ± 1.5</td>
<td>$&lt;0.001**$ (MW)</td>
</tr>
<tr>
<td><strong>Smoker (% frequency)</strong></td>
<td>10%</td>
<td>27%</td>
<td>0.33 (FE)</td>
</tr>
</tbody>
</table>

**Table 12 Summary of demographic and clinical variables of subjects (PPD, probing pocket depth).** Data shown represent mean ± standard deviation. Tests of significance performed were $t$-test, $MW$ (Mann-Whitney $U$-test), $FE$ (Fisher’s exact test) according to whether or not data were normally distributed. *$p<0.05$, **$p<0.001$
Figure 4 Examples of radiographs taken from subjects enrolled in the study. Top picture: Implants with peri-implantitis showing saucer shaped bone loss commonly found with peri-implantitis. Top row left to right patient 13, 8, 11, 1 lower row 12, 14 17, 6. Bottom picture: Healthy implants showing no loss of crestal bone around implants. Left patient 19, Top right 18, bottom right 9.
2.4.2 MICROBIOLOGICAL OBSERVATIONS

2.4.2.1 QUANTITATIVE CULTURE ANALYSIS

Table 13 compares the mean CFU/ml in healthy and peri-implantitis sites. The raw data for CFU/ml for every sample can be found in Appendix 4 - Table 28

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Peri-implantitis</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean aerobic CFU/ml (S.D)</td>
<td>2.22 x 10^6 (3.3 x 10^6)</td>
<td>1.16 x 10^7 (3.6 x 10^7)</td>
<td>0.9</td>
</tr>
<tr>
<td>Mean anaerobic CFU/ml (S.D)</td>
<td>5.89 x 10^6 (8.9 x 10^6)</td>
<td>3.62 x 10^7 (6.6 x 10^7)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Table 13 Mean (S.D.) of CFU/ml (colony forming units/ ml) around healthy implants (n=10) and in peri-implantitis (n=11) after being cultured aerobically for 5 days and anaerobically for 10 days. *T-test of log transformation of the data

The average CFU from peri-implantitis samples was generally 10 times more than that found around healthy implants and the mean number of bacteria cultured under anaerobic conditions showed approximately 3 times more bacteria than when cultured aerobically, but no significant differences could be detected (Table 13, Figure 5).

![Figure 5 Difference in mean total viable counts (CFU/ml) between healthy implants (n=10) and those with peri-implantitis (n=11)]
2.4.2.2 Cultivable Taxa from Healthy and Failing Dental Implants

Due to an error during laboratory procedures, sequences from sample 2 (healthy) were lost and were not considered in subsequent sequencing or statistical analyses. Thus, from 20 samples (9 healthy and 11 failing), a total of 1670 sequences were identified (80 – 93 sequences per sample) representing 5 phyla: Firmicutes (57%), Actinobacteria (17%), Proteobacteria (11%), Bacteroidetes (10%), and Fusobacteria (5%). The number of bacterial species identified from the healthy group were 80 species (from 753 sequences) and those from the peri-implantitis group were 89 species (from 917 sequences). The relative proportion of each species in each group can be seen in figures 6 and 8. Their corresponding phylogenetic trees (figures 7 and 9) show the evolutionary relationships between the bacterial species detected in both groups.
Figure 6 Prevalence of taxa identified in 9 healthy implant samples
Figure 7 Neighbour joining tree of bacterial species & phyla detected in healthy implants (n=9) constructed using the Jukes Cantor method.
Figure 8 Prevalence of taxa identified in 11 peri-implantitis samples
Figure 9 Neighbour joining tree of bacterial species & phyla detected in peri-implantitis (n=11) constructed using the Jukes Cantor method.
A total of 45 genera were identified, 41 in peri-implantitis and 31 in healthy. The major genera that made up the subgingival microbiota around implants was comprised of the following Actinomyces (oris, naeslundii, meyeri and israelii), Streptococcus (oralis/ mitis/ pneumoniae, constellatus, anginosus, intermedius, sanguinis, gordonii, parasanguinis II, sp. HOT-071, salivarius and crista), Veillonella (parvula/ dispar and atypica), Fusobacterium (nucleatum), Neisseria (flavescens, subflava, elongata and bacilliformis), Parvimonas (micra and sp. HOT-111), Rothia (dentocariosa, mucilaginosa, sp. HOT-188), Filifactor (alocis), Prevotella (nigrescens and intermedia), Gemella (haemolysans and morbillorum), Capnocytophaga (sp. HOT-412), Haemophilus (parainfluenzae), Eikenella (corrodenes), Porphyromonas (gingivalis) and Pseudoramibacter (alactolyticus). A comparison between their distribution in the healthy and peri-implantitis samples can be found in figure 10.

Figure 10 Graph showing the percentage of samples in which most frequently observed species were found. *p < 0.05, **p < 0.01, ***p < 0.001 Fisher’s exact test
Overall *S. constellatus* was the most abundant sequence in terms of how many times it was isolated (141/1670 sequences i.e. 8.5% of all sequences) and was present in 11 out of the 20 sampled sites (55%). Species that were found in most sampling sites were *A. oris/ naeslundii* (85% of sampled sites and 8.3% of isolates), *F. nucleatum* (75% of sampled sites and 3% of isolates) and *S. mitis/ oralis/ pneumoniae* (60% of sampled sites and 6% of isolates).

In sites with peri-implantitis, *F. alocis* (*p<0.001*), *S. constellatus*, *P. micra* and *A. meyeri* (*p<0.05*) were significantly more prevalent than in healthy sites. *F. alocis* was detected in nearly all (75%) the failing samples, yet in none of the healthy sites. On the other hand, *V. parvula* (*p<0.01*) and *N. elongata* (*p<0.05*) were more likely to be isolated in the healthy sites (Table 14)

<table>
<thead>
<tr>
<th>Species</th>
<th>Diagnosis</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy</td>
<td>Peri-implantitis</td>
</tr>
<tr>
<td><em>Veillonella parvula</em></td>
<td>0.89 ± 0.33</td>
<td>0.27 ± 0.47</td>
</tr>
<tr>
<td><em>Streptococcus constellatus</em></td>
<td>0.22 ± 0.44</td>
<td>0.82 ± 0.41</td>
</tr>
<tr>
<td><em>Parvimonas micra</em></td>
<td>0.22 ± 0.44</td>
<td>0.82 ± 0.41</td>
</tr>
<tr>
<td><em>Filifactor alocis</em></td>
<td>ND</td>
<td>0.73 ± 0.47</td>
</tr>
<tr>
<td><em>Neisseria elongata</em></td>
<td>0.44 ± 0.52</td>
<td>ND</td>
</tr>
<tr>
<td><em>Actinomyces meyeri</em></td>
<td>ND</td>
<td>0.45 ± 0.5</td>
</tr>
</tbody>
</table>

**Table 14** Means ± standard deviations of species that were significantly different between both groups *Fisher’s exact test (ND, not detected)*

Organisms that were only detected around healthy implants and those only in peri-implantitis are summarized in the following table (Table 15). Most of these did not reach statistical significance.

<table>
<thead>
<tr>
<th>Healthy</th>
<th>Peri-implantitis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinomyces georgiae</em></td>
<td>HOT-617</td>
</tr>
<tr>
<td><em>Actinomyces johnsonii</em></td>
<td>HOT-849</td>
</tr>
<tr>
<td><em>Actinomyces massiliensis</em></td>
<td>HOT-852</td>
</tr>
<tr>
<td><em>Actinomyces radicidentis</em></td>
<td>HOT-746</td>
</tr>
<tr>
<td><em>Capnocytophaga gingivalis</em></td>
<td>HOT 337</td>
</tr>
</tbody>
</table>
### Table 15 Summary of species found ‘only in healthy’ and ‘only in peri-implantitis.

Those which reached statistical significance are indicated in red.

<table>
<thead>
<tr>
<th>Species</th>
<th>HOT Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capnocytophaga sputigena</td>
<td>775</td>
</tr>
<tr>
<td>Campylobacter showae</td>
<td>763</td>
</tr>
<tr>
<td>Capnocytophaga granulosa</td>
<td>325</td>
</tr>
<tr>
<td>Gemella sanguinis</td>
<td>757</td>
</tr>
<tr>
<td>Granulicatella adiacens</td>
<td>534</td>
</tr>
<tr>
<td>Kingella oralis</td>
<td>706</td>
</tr>
<tr>
<td>Lachnospiraceae [G-1] sp.</td>
<td>107</td>
</tr>
<tr>
<td>Micrococcus sp.</td>
<td></td>
</tr>
<tr>
<td>Neisseria sp.</td>
<td>016</td>
</tr>
<tr>
<td>Neisseria elongata</td>
<td>598*</td>
</tr>
<tr>
<td>Bacteroides [G-1] (Prevotella) sp.</td>
<td>272</td>
</tr>
<tr>
<td>Prevotella denticola</td>
<td>291</td>
</tr>
<tr>
<td>Prevotella maculosa</td>
<td>289</td>
</tr>
<tr>
<td>Prevotella multiformis</td>
<td>685</td>
</tr>
<tr>
<td>Prevotella oulorum</td>
<td>288</td>
</tr>
<tr>
<td>Prevotella salivae</td>
<td>307</td>
</tr>
<tr>
<td>Pseudomonas psychrotolerans</td>
<td></td>
</tr>
<tr>
<td>Rothia aeria</td>
<td>188</td>
</tr>
<tr>
<td>Selenomonas sp.</td>
<td>137</td>
</tr>
<tr>
<td>Selenomonas noxia</td>
<td>130</td>
</tr>
<tr>
<td>Selenomonas sputigena</td>
<td>151</td>
</tr>
<tr>
<td>S. infantis</td>
<td>638</td>
</tr>
<tr>
<td>S. mitis bv 2</td>
<td>398</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>601</td>
</tr>
<tr>
<td>Prevotella intermedia</td>
<td>643</td>
</tr>
<tr>
<td>Prevotella sp.</td>
<td>317</td>
</tr>
<tr>
<td>Prevotella sp.</td>
<td>782</td>
</tr>
<tr>
<td>Prevotella sp.</td>
<td>820</td>
</tr>
<tr>
<td>Propionibacterium acnes</td>
<td>530</td>
</tr>
<tr>
<td>Propionibacterium propionicum</td>
<td>739</td>
</tr>
<tr>
<td>Slackia exigua</td>
<td>602</td>
</tr>
<tr>
<td>Solobacterium moorei</td>
<td>678</td>
</tr>
<tr>
<td>Streptococcus sobrinus</td>
<td>768</td>
</tr>
<tr>
<td>Streptococcus vestibularis</td>
<td>021</td>
</tr>
</tbody>
</table>

| Bulleidia extracta            | 603      |
| Campylobacter gracilis        | 623      |
| Capnocytophaga sp.            | 380      |
| Cardiobacterium valvulum      | 540      |
| Dialister invisus             | 118      |
| Dialister pneumosintes        | 736      |
| Eubacterium [XI][G-6] nodatum | 694      |
| Eubacterium [XI][G-7] yurii   | 377      |
| Filifactor alocis             | 539      |
| Fusobacterium necrophorum     | 690      |
| Haemophilus haemolyticus      | 535      |
| Lactobacillus [XVII] catenaformis | 569  |
| Lactobacillus delbrueckii     |          |
| Lactobacillus gasseri         | 615      |
| Lactobacillus parabuchneri    |          |
| Lactobacillus paracasei       | 716      |
| Lactobacillus salivarius      | 756      |
| Micrococcus luteus            |          |
| Mogibacterium timidum         | 042      |
| Olsenella ali                 | 038      |
| Peptoniphilus sp.             | 836      |
| Peptostreptococcus stomatis   | 112      |
| Prevotella baroniae           | 553      |
| Prevotella buccae             | 560      |
| Prevotella intermedia         | 643      |
| Prevotella sp.                | 317      |
| Prevotella sp.                | 782      |
| Prevotella sp.                | 820      |
| Propionibacterium acnes       | 530      |
| Propionibacterium propionicum | 739      |
| Slackia exigua                | 602      |
| Solobacterium moorei          | 678      |
| Streptococcus sobrinus        | 768      |
| Streptococcus vestibularis    | 021      |
A summary of the overall results (proportion and phylogenetic relationship) of the subgingival peri-implant microbial community (phyla, genera and species) detected in peri-implantitis and in health is illustrated in figure 11.

Figure 11 Phylogenetic relationship (N-J) of all subgingival peri-implant bacterial species identified and their overall proportional abundance (%) in health (green) and in peri-implantitis (red). Created using the Interactive Tree of Life (ITOL). (*p<0.05, **p<0.01, ***p<0.001 Fisher’s exact test) (n=20)
2.4.2.3 Results from the sialidase test

In the present study, when sequences were identified as *S. constellatus* using the forward primers, blasting results from the same isolates that were sequenced using the reverse primers, identified them to be *S. intermedius*. The sialidase test showed that *S. constellatus* and *S. intermedius* were correctly identified using the 16S forward primers, but could not be differentiated using the reverse primers.

2.4.2.4 Results from Met-G sequencing

Met-G sequencing revealed that 32 out of the 36 strains were *A. oris* (Table 16). The rest were *A. naeslundii* and were all from Sample 4, which was from an implant with peri-implantitis. This was different to the results obtained from 16S rRNA (where most were identified as *A. naeslundii*) supporting the evidence that 16S rRNA PCR is not the best method to reliably differentiate between these 2 species (Henssge et al., 2009). Due to time constraints however, this method of differentiation was limited to samples 4, 5, 6 and 7. In this study, *A. naeslundii* and *A. oris* were grouped into the same category *A. naeslundii/ oris*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Met – G Identification</th>
<th>16S Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>7o5_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>7o11_MR</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>7a15_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>7o16_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>7o22_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>7o25_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>7a27_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>7o32_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>7o33_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>7o37_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>7o39_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>7o40_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>4a7_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>4a33_MR</td>
<td><em>Actinomyces naeslundii</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>4o4_MF</td>
<td><em>Actinomyces naeslundii</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>4o20_MR</td>
<td><em>Actinomyces naeslundii</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>4o22_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>4o23_MR</td>
<td><em>Actinomyces naeslundii</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
</tbody>
</table>
Table 16 Identification of *Actinomyces* using Met-G and 16S Universal primers

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Species 1</th>
<th>Species 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>4o25_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>4o27_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces naeslundii</em></td>
</tr>
<tr>
<td>5o3_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>5o13_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>5o15_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces radicidentis</em></td>
</tr>
<tr>
<td>5o16_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>5o20_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>5o22_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>5o23_MR</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>5o28_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>5o31_MR</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>5o32_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>5a37_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>5o47_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>6o8_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>6o14_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>6o16_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
<tr>
<td>6o46_MF</td>
<td><em>Actinomyces oris</em></td>
<td><em>Actinomyces sp.</em></td>
</tr>
</tbody>
</table>

Diagram:

```
  | Actinomyces naeslundii (reverse metG primer)
  | Actinomyces oris (reverse metG primer)
  | 4a33 MR
  | 4o4 MR
  | 4o23 MR
  | 4o20 MR
  | 5o31 MR
  | 5o23 MR
  | 7a22 MR
  | 7a27 MR
  | 7o11 MR
```

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2.5 DISCUSSION

Peri-implantitis is becoming an increasingly common occurrence. At present, the management of the condition is often difficult and therapeutic success is unpredictable (Claffey et al., 2008, Heitz-Mayfield and Mombelli, 2014, Kotsovilis et al., 2008, Renvert et al., 2008). The current consensus is that peri-implantitis is a bacterially-induced inflammation of the supporting peri-implant tissues (Lindhe et al., 2008). Therefore, it would be valuable to obtain a comprehensive picture of the microbial composition at peri-implantitis sites since this could provide useful information for the development of effective therapeutic approaches.

When the protocol for this study was proposed in November 2009, there were no studies that had explored the microbiota using an open-ended approach. Previous studies focused mainly on specific microorganisms especially those designated as 'periodontal pathogens', and were not designed to detect other species. These studies used culture methods, microscopy, (Botero et al., 2005, Leonhardt et al., 1999, Listgarten and Lai, 1999, Mombelli et al., 1987, Rams et al., 1990, Tabanella et al., 2009, Alcoforado, 1991 #7), DNA-DNA checkerboard hybridization (Salcetti et al., 1997, Heuer et al., 2007, Shibli et al., 2008) and 16S rRNA gene sequencing utilizing species specific molecular probes (Heuer et al., 2007, Tabanella et al., 2009, Ferreira et al., 2009).

Although our study used a preliminary culture method, which has its limitations of not detecting the unculturable bacteria, it was our aim to obtain a clearer picture of the microbial diversity around implants by utilizing a 16S rRNA gene sequence analysis to identify the species that were cultured. This has become a useful approach to assess the phylogenetic and taxonomic diversity of bacterial isolates (Tamura et al., 2013b) and results were confirmed through Gram staining and biochemical testing when necessary.

2.5.1 MICROBIAL DIVERSITY AROUND DENTAL IMPLANTS

During the past two years, four studies have been published that have employed non-specific open-ended approaches to determine the microbial diversity around implants (da Silva et al., 2013, Koyanagi et al., 2013, Kumar et al., 2012, Tamura et al., 2013b) and these are summarized in Table 17. This discussion will focus primarily on the comparison of the results of the present study with these recent published investigations.
<table>
<thead>
<tr>
<th>Study</th>
<th>Method</th>
<th>Main Findings</th>
</tr>
</thead>
</table>
| (Tamura et al., 2013b)                     | Anaerobic culture and identification using 16S rDNA sequencing | **Genera:**  
**Peri-implantitis (Pi):** *Streptococcus* (34%), *Eubacterium* (13%), *Prevotella* (10%), *Actinobacteria* (6%), *Fusobacteria* (4%)  
**Healthy (H):** *Streptococcus* (45%), *Actinobacteria* (14%), *Veillonella* (14%), *Propionibacter* (8%)  
**Species**  
**Peri-implantitis:** 69 species (10x higher CFU count)  
*F. alocis, P. micra, P. intermedia, E. nodatum, E. brachy, S. exigua, Parascardovia denticolens, F. nucleatum* were the predominant obligate anaerobes  
**Healthy:** 53 species  
*Veillonella, P. acnes, P. alactolyticus, P. micra* were the predominant obligate anaerobes: |
| (da Silva et al., 2013)                     | 16S rRNA clone-sequencing                                   | **Genera:** 43 Total Genera  
**Pi** *Campylobacter, Desulfbobulus, Dialister, Eubacterium, Filifactor, Mitsukella, Parvimonas, Porphyromonas, Pseudoramibacter, Fusobacterium*  
**H:** *Actinomyces, Atopobium, Gemella, Kingella, Rothia, Veillonella*  
**Species** 1387 clones  
**Pi:** *F. alocis, P. micra, P. intermedia, C. gracilis, D. invisus, F. nucleatum, Streptococcus sp. HOT-064, Eubacterium infirnum, Mitsukella sp. HOT-131*  
**H:** *V. dispar, G. adiacens, S. mitis, A. meyeri* |
<table>
<thead>
<tr>
<th>Study</th>
<th>Methodology</th>
<th>Study Population</th>
<th>Summary</th>
</tr>
</thead>
</table>
| (Koyanagi et al., 2013) | 16S rRNA gene clone library       | 6 patients (6 failing implants, 6 periodontitis sites)                            | Total: 799 clones, 333 different taxa

**Pi**: 192 taxa, **Pdtitis**: 148 taxa

- Pi showed greater diversity than pdtitis.
- Microbial population was different from pdtitis
- *F. nucleatum* (most abundant sequence)
- *Dialister* and *Eubacterium* higher prevalence in Pi than pdtitis
- *P. micra*, *P. stomatitidis*, *P. alactolyticus*, *S. moorei* were only present in Pi, not in pdtitis
- Prevalence of periodontopathogenic bacteria was not high

**Note:** Only reports presence/absence. No mention of proportions or statistical significance.

<table>
<thead>
<tr>
<th>Study</th>
<th>Methodology</th>
<th>Study Population</th>
<th>Summary</th>
</tr>
</thead>
</table>
| (Kumar et al., 2012) | 16S pyrosequencing                   | 10 subjects; healthy implants 10 subjects; peri-implantitis 10 subjects; pdl healthy 10 subjects; periodontitis | Healthy Implants:

- Microbial profile of healthy implants was more diverse than failing.
- Had the highest levels of Gram-negative anaerobes and lowest Gram-positive aerobes
- *Leptotrichia*, *Propionibacter* and *Veillonella* were significantly greater than in Pi.

Peri-implantitis:

- Pi is a simple, heterogeneous infection, had the fewest species of all.
- Species belonging to 4 genera made up 75% of the Pi microbial community.
- *Butyrivibrio*, *S. mutans*, *Actinomyces*, non-*mutans* streptococci, *Campylobacter* greater proportion than healthy

Significant differences between peri-implant and periodontal microbiota

**Table 17 Summary of recent studies describing the microbiota around implants. Pi, peri-implantitis; H, healthy; pdtitis, periodontitis;**
In the present study, peri-implantitis was accompanied by an increased bacterial load. This is in agreement with the study by Tamura et al. (Tamura et al., 2013b). The remainder of the studies did not comment on the bacterial load.

The distribution of phyla were comparable between peri-implantitis and health, with *Firmicutes*, which are mainly Gram-positive making up the greatest proportion. Similarly, *Firmicutes* made up the greatest proportion of phyla in the other studies (da Silva et al., 2013, Koyanagi et al., 2013, Kumar et al., 2012, Tamura et al., 2013b). However Silva et al. found that *Bacteroidetes* were significantly raised in peri-implantitis while *Actinobacteria* were significantly less. The other studies did not compare between the phyla. It is worth mentioning that some other phyla, particularly those considered difficult to culture or unculturable, namely TM7, *Synergistetes* and *Spirochaetes* were not detected in our study, but were detected in the studies by Kumar, da Silva and Koyanagi. These were detected in both peri-implantitis and healthy groups, however their proportions were very small (< 1%) and no significant differences were detected between groups (Kumar et al., 2012, da Silva et al., 2013, Koyanagi et al., 2013).

The present study has demonstrated that in these partially dentate subjects, a complex and heterogeneous subgingival microbiota is generally found around dental implants that have been in function for more than one year. These findings are in agreement with the majority of the other studies (Tamura et al., 2013b, da Silva et al., 2013, Koyanagi et al., 2013). On the other hand our findings diverge from those by Kumar et al. (Kumar et al., 2012) who have reported that peri-implantitis was a simple infection with relatively low diversity and that only 4 genera made up 75% of the peri-implantitis microbial community in each individual. The composition they reported for the peri-implant microbiome was also surprising, in that the genera *Streptococcus* and *Actinomyces* were more prevalent in the peri-implantis group.
2.5.2 The predominant micro-organisms in peri-implantitis and in health

When the flora of the two groups was compared at the species level, there seemed to be a shift towards a proportionally more ‘pathogenic flora’ in the peri-implantitis sites. It was noted that some potentially important organisms were consistently found in higher proportions in the peri-implantitis group and vice-versa.

*F. alocis, S. constellatus, P. micra* and *A. meyeri* were predominantly more in the peri-implantitis group. These findings are supported by the results of Tamura et al., da Silva et al. and Koyanagi et al. (Tamura et al., 2013b, da Silva et al., 2013, Koyanagi et al., 2013, Kumar et al., 2012). However there was no mention of *A. meyeri* except in the study by Tamura et al. (Tamura et al., 2013b), in which contrary to our findings, was predominant in the healthy sites.

*V. parvula/dispar* and *N. elongata* on the other hand were predominantly more in the healthy group. This is in accordance with other studies that have designated the same species to be health-compatible periodontal bacteria (Tamura et al., 2013b, da Silva et al., 2013, Kistler et al., 2013).

Other species that were significant in peri-implantitis in the other studies (Table 17) e.g. species like *Dialister invisus, Campylobacter gracilis, Eubacterium nodatum, P. intermedia, Peptostreptococcus stomatitis, Solobacterium moorei* and *Slackia exigua* were also isolated solely from peri-implantitis samples in our study, however they were not abundant enough to reach a statistically significant level. Similarly, the other studies have shed the light on the importance of *Kingella oralis* and *Gramulicatella adiacens* in healthy sites. Again these species were only found in healthy sites in our study, but their proportions were not sufficiently high to reach a significant level.
CHAPTER 3  Biofilm Formation on Titanium Implant Surfaces – an in vitro study (study 2)
3.1 **INTRODUCTION**

Most bacteria live in a biofilm state. This enhances their survival, metabolism, and propagation especially under adverse conditions. The formation of a biofilm depends on two main factors, the microorganisms present in the environment and the surface on which this biofilm is formed (Costerton et al., 1999, Mah and O'toole, 2001).

3.1.1 **EARLY COLONIZERS OF ORAL BIOFILMS**

Oral streptococci are early colonizers of intra-oral biofilms, making up 80% of the early biofilm constituents (Rosan and Lamont, 2000). They play an important role in biofilm progression and the resultant biofilms maybe related to health or disease depending on the bacterial composition. They are part of the genus *Streptococcus* and are generally referred to as *viridans* streptococci, although *viridans* streptococci also contain other members that are not found in the oral cavity. As the name implies, they inhabit predominantly the oral cavity and upper respiratory tract of humans and animals (Aas et al., 2005).

They are divided into five different groups (1) *Mutans* group (prominent members are *S. mutans* and *S. sobrinus*) (2) *Salivarius* group (*S. salivarius*) (3) *Anginosus* group (*S. anginosus* and *S. intermedius*) (4) *Sanguinis* group (*S. sanguinis* and *S. gordonii*) and (5) *Mitis* group (*S. mitis* and *S. oralis*) (Whiley and Beighton, 1998).

3.1.1.1 **STREPTOCOCCUS ORALIS**

*S. oralis* are facultatively anaerobic gram-positive, non-spore forming, non-motile cocci approximately 1 µm in diameter that grow in short or long chains. They utilize intracellular glycogen to form lactic acid, form alpha haemolytic colonies on blood agar (0.7-1.2 mm in diameter) and G+C content is 41% mol % (van Houte and Jansen, 1970).

They are normal commensals of the oral flora and are important pioneer bacteria in the initial attachment of multispecies biofilms on oral surfaces. They are not usually pathogenic, but can be opportunistic pathogens and are associated with subacute bacterial endocarditis and septicaemia in immunocompromised patients (Kreth et al., 2009).

*S. oralis* is closely related phylogenetically to the other *viridans* streptococci, *S. mitis*, *S. pneumoniae* and *S. pseudopneumoniae*. Despite differences in pathogenic potential, they
share over 99% 16S rRNA sequence identity and are difficult to differentiate using conventional biochemical tests (Do et al., 2009, Park et al., 2010, Whatmore et al., 2000).

3.1.2 IMPLANT SURFACES

Titanium implant surfaces provide an ideal biological niche for the development of a biofilm. The surfaces play a crucial role in biological interactions, as they are the only part in contact with the bio-environment and hence influence the implant/ tissue interface.

The first implants (known as machined or turned implants) had a moderately smooth machined surface. Surface coatings have since been developed to promote and enhance bone apposition and decrease the healing time for osseointegration. The modifications used, whether being an altered surface chemistry and/or an increase in surface texture may however influence biofilm formation if the implant were to become exposed, as in the case of peri-implantitis (Palmquist et al., 2010b).

Currently, there is controversy as to which implant surfaces and modifications affect biofilm formation (Zhao et al., 2005, Lin et al., 2013). There is also controversy regarding if different implant surfaces affect the progression of peri-implantitis (Heitz-Mayfield, 2008) and whether the addition of chemical or bioactive molecules may help in reducing this.
3.2 Study 2 Aims and Objectives

Aim

To assess whether there was a difference in biofilm formation on different titanium implant and abutment surfaces.

Objectives

1. To use identified relevant microorganism(s) in an in vitro biofilm model system.

2. To investigate characteristics of dental implant surfaces that could influence the formation of a biofilm.

3. To compare differences in volume, quantity and viability of biofilms on the different surfaces.
3.3 Materials and Methods

In order to enable us to study the development of a biofilm on different implant surfaces, and so that we could subsequently study its ease of removal from various implant surfaces, it was necessary to develop a simplified and standardized in vitro model. Hence a single species biofilm model was developed, made up of a representative species identified in chapter 2. Initial attempts to develop a multispecies biofilm yielded inconsistent and unpredictable results. The results of these initial attempts are presented in Appendix 5.

3.3.1 Choice of Organisms

After analysis of the 21 patient samples (see chapter 2), *S. oralis* was chosen to develop a single species biofilm since it represented an abundant species isolated from both peri-implantitis and healthy implant sites. The strain was identified using 16S rRNA sequencing analysis and Gram staining.

3.3.2 Growth Curves for Streptococcus Oralis

To give a general idea of the growth patterns of *S. oralis*, growth curves were monitored using the Flouroskan plate reader. A suspension was prepared in mFUM, then 200µl were dispensed in replicates of 4. The following procedure was programmed into the reader: temperature was set at 37 ºC and plates were shaken and absorbance measured every 15 minutes at a wavelength of 600 nm for 48 hours.

3.3.3 Strains and Inoculum Preparation

A strain identified as *S. oralis* | Oral Taxon 707 | Strain ATCC 35037/ *S. mitis* | Oral Taxon 677 | Strain ATCC 49456 (Human Oral Microbiome Database (HOMD)) was obtained from the frozen preserves of sample number 10. It was grown anaerobically on FAA supplemented with 5% (v/v) defibrinated horse blood for 2 days. A loopful of each bacterial strain was then inoculated into 5 ml of sterilized modified fluid universal medium (mFUM) containing 0.2% glucose and further incubated anaerobically at 37ºC.

After 4 hours (mid-exponential phase), 1ml of the pre-cultures were drawn into a cuvette and Optical Density (O.D.) was measured at a wavelength of 600 nm using a spectrophotometer (Cecil Super Aquarius CE 9200 Double Beam UV/VIS
Spectrophotometer). Bacterial suspensions were adjusted for an O.D. of 0.5 ± 0.05 (seed cultures).

3.3.4 Titanium surfaces

Titanium discs were produced at Astra Tech (Dentsply Implants, Astra Tech, Mölndal, Sweden). The discs, which were used as a substrate for biofilm formation, were round in shape (6.5 mm in diameter, 2 mm thick and 33.2 mm² in surface area) and were made out of commercially pure (CP) grade IV titanium, except surface number 7873, which was made of Grade V CP Ti. They were modified in four different ways to create four different surfaces (Table 18 and Appendix 6). The surfaces were coded and researchers were blinded to what the surfaces were until after completion and analysis of all experiments. 6905 and 7874 were characterized as being moderately rough (Albrektsson and Wennerberg, 2004b). 6905 was produced by blasting with titanium dioxide particles, while 7874 was produced by first blasting with titanium dioxide particles then subsequently treating the surface with hydrofluoric acid according to the OsseoSpeed manufacturing procedure. Surfaces 7676 and 7873 were characterized as being smooth (Albrektsson and Wennerberg, 2004b) and were produced by machining to achieve a final surface roughness of Sa ≈ 0.2 µm.

Palmquist et al., (Palmquist et al., 2010b) have proposed that sterilization and storage in a sterile package may influence the surface of a dental implant, suggesting that this occurs via the transfer of molecules from the packaging material to the implant surface. This means that in order to produce reproducible implant surfaces, all aspects of the manufacturing process and resultant logistics need to be carefully controlled. In our study this was strictly adhered to. The discs were provided in exactly the same manner by which Astra implants are packaged and stored. They were degreased, cleaned, double packaged and fixed to a mount, and thus could be handled aseptically without any need to touch or contaminate them. Furthermore, they received the same rigorous sterilization process (beta-sterilized by >25<75kGy) that the Astra dental implants are subjected to. Although not rough and smooth in the true sense of the words, for the purpose of this study, surfaces 6905 and 7874 will be referred to as rough, whilst 7873 and 7676 will be referred to as smooth.
### Table 18 Surfaces and surface modifications investigated in this study

<table>
<thead>
<tr>
<th>Surface reference number</th>
<th>Surface Modification to the titanium discs</th>
</tr>
</thead>
<tbody>
<tr>
<td>6905 TiOblast</td>
<td>Titanium discs blasted using titanium dioxide particles</td>
</tr>
<tr>
<td>7874 Test, Tioblast + HF</td>
<td>Titanium discs blasted with titanium dioxide particles then acid-etched using HF acid according to a proprietary process of Astra</td>
</tr>
<tr>
<td>7676 Test machined</td>
<td>Machine produced</td>
</tr>
<tr>
<td>7873 Test Ti Gr.5</td>
<td>Machine produced</td>
</tr>
</tbody>
</table>

#### 3.3.5 Growth Of the Biofilm

The titanium discs were placed in a 24-well polystyrene cell culture plate and pre-incubated for 6 hours with 1ml of mFUM (modified Fluid Universal Medium) to precondition the discs. The mFUM was originally described by Gmür and Guggenheim (Gmür and Guggenheim, 1983) in 1983. It contains (per litre of distilled water) 10 g tryptone, 5 g yeast extract, 2 g glucose, 0.4 ml stock hemin (5 mg/l) solution, 0.5 ml stock menadione (2 mg/l) solution, 0.5 g cysteine hydrochloride, 0.1 g dithiothreitol, 2.9 g NaCl, 0.5 g Na₂CO₃, 1 g KNO₃, 0.45 g K₂HPO₄, 0.45 g KH₂PO₄, 0.9 g (NH₄)SO₄, MgSO₄,7H₂O and finally heat-inactivated, filter-sterilized, horse serum.

100 µl of seed cultures were added to the discs with the mFUM. The growth medium was replenished every 24 hours by aspirating the spent medium carefully from the wells and the addition of fresh medium taking care not to disturb the early biofilm. This was carried out with the plates still in the anaerobic incubator and was repeated for 4 days (i.e. 3 media changes) after which the plates were taken out of the anaerobic chamber and prepared for analysis (Figure 12).
Figure 12 Schematic timeline of the biofilm experiment

**Saturday**
Serains grown anaerobically for 48 hours on FAA

**Monday 9:00 am (Precultures)**
Inoculate mFUM and incubate for 6 hours

**Monday 3:00 pm (seed cultures)**
Optical Denisty adjusted to 0.5
Add 100ul of seed cultures to discs

**Tuesday 3:00 pm**
Media Change 24 hours

**Wednesday 3:00 pm**
Media Change 48 hours

**Thursday 3:00 pm**
Media Change 72 hours

**Friday 3:00 pm**

a) Biofilms stained for confocal

b) Biofilms harvested to determine bacterial counts
3.3.6 **Examination of the Biofilm**

3.3.6.1 **Harvesting of the Biofilm**

The discs were dip-washed twice and swirled gently in sterile Phosphate Buffered Saline (PBS) to remove the loosely adherent bacteria. Each disc was then transferred to a sterile 7ml bijou containing 1ml of Fastidious Anaerobic Broth (FAB) and a few sterile glass beads. It was vortexed vigorously for 30 seconds to disrupt the biofilm and harvest adherent bacteria, after which the suspension was serially diluted ($10^0$-$10^5$). 100 µl of each diluted sample was plated on FAA and the plates were incubated anaerobically at 37ºC. Tests were performed in duplicates and after 5 days, the numbers of CFUs per mL were counted.

3.3.6.2 **Confocal Laser Scanning Microscopy (CLSM)**

*Staining of the Biofilm*

To enable us to assess bacterial vitality, the biofilms were stained with a LIVE/DEAD® BacLight™ Bacterial Viability Kit for microscopy and quantitative assays (Molecular Probes, Invitrogen, UK). These kits utilize a STYO® 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. These stains differ in their spectral characteristics and in their ability to penetrate healthy bacterial cells. With an appropriate mixture of the STYO® 9 and propidium iodide stains, bacteria with intact cell membranes are stained fluorescent green, whereas those with damaged bacterial cell membranes stain fluorescent red, whilst the background remains virtually non-fluorescent.

After dyes were left to warm to room temperature, 2 µl of component A (STYO® 9) and 2 µl of component B (propidium iodide) were added to 4 ml of PBS and kept in a bijou covered with tin foil to protect against light. For each biofilm-covered disc, 500 µl of the dye mixture was placed in a dry well of a 24-well plate. The disc was immersed inside this and the plate was covered with tin foil and left aside for 15 minutes. After staining, the disc was dipped once more in PBS and placed inverted in a glass-bottomed 35 mm diameter Petri dish (MatTek Corporation, Ashland, USA) to be mounted on the CLSM.
**CLSM Imaging**

Confocal imaging of the biofilms was accomplished by means of a Leica inverted CLSM (TCS SP2; Leica Microsystems, Wetzlar, Germany) equipped with an x63 oil immersion lens (HCX PL APO CS; NA = 1.4, Leica Microsystems) with a numerical aperture of 1.4. An Ar-Kr Laser was used with an excitation wavelength of 488 nm and 569 nm for STYO® 9 and propidium iodide respectively. A negative control was used to set the microscope parameters to avoid reflection of the titanium itself as much as possible. After the gain was adjusted to reduce reflection, the lens was moved away from the surface, until reflection became minimal. This z-level was recorded and used as a starting level for each set of biofilm images.

The biofilm structure was examined at 5 random points (5 stacks) as a series of horizontal optodigital sections, each 2 µm thick (z-step) for an overall height of 20 µm (i.e. 10 two-dimensional (2-D) images per stack). Images were obtained at a zoom factor of 2.0, a pixel resolution of 0.42 µm pixel\(^{-1}\) and a field resolution of 512 x 512. The mean of the 50 measurements was taken for statistical analysis.

Digital images were transformed to the tiff format and processed using the bioImage_L software (Chavez de Paz, 2009). Using this software, cell counting, biovolume, mean height, and substratum coverage can be analyzed.

All experiments (confocal and culture counts) were carried out 10 times for each surface using independent bacterial cultures.

### 3.3.6.3 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS)

Topographic characterization of the surfaces was carried out using scanning electron microscopy (SEM) before and after biofilm formation. In addition, chemical analysis of the surfaces was performed using Energy-dispersive X-ray spectroscopy (EDS).
PREPARATION OF PRISTINE SURFACES FOR SEM

The original titanium surfaces were analyzed (SEM & EDS) with no coating. They were mounted on 12 mm pin stubs (Hitachi High Technologies, Berkshire, UK) with double sided adhesive carbon tab (TAAB Laboratories Equipment, Berkshire, UK). No fixing, osmication or dehydration was necessary. Discs were directly mounted and carbon coated (Emitech K250 sputter coater).

PREPARATION OF SURFACES WITH BIOFILM FOR SEM

The biofilm samples were first fixed for 1 hour at 4°C with 2.5% glutaraldehyde in 0.1m Phosphate buffer (pH 7.2), then washed twice for 5 minutes in 0.1M Phosphate buffer. After which they were osmicated at 4°C for 40 minutes (1% osmium tetroxide) and then dehydrated through a series of graded ethanol (30%, 50%, 70% and 95% ethanol for 5 minutes each and 3 times in 100% ethanol for 10 minutes each).

They were then transferred to 100% ethanol in critical point drying holders and were critical point dried using carbon dioxide in a Polaron E3000 critical point dryer (Quorum Technologies Ltd, West Sussex, UK).

Finally, the samples were sputter coated with gold in an Emitech K550X sputter coater (Quorum Technologies Limited, West Sussex, UK) using a 45mA current for 1 minute and 40 seconds and were then mounted as described above for the pristine surfaces.

Surfaces were examined and representative images were recorded using a FEI Quanta™ 200F field emission scanning electron microscope fitted with an EDAX Genesis x-ray microanalysis system.

ANALYSIS USING EDS

EDS was performed in order to test differences in chemical composition between the surfaces in their pristine state and after biofilm decontamination. This is accomplished through the interaction of primary electron beams from the SEM with surface atoms producing characteristic measurements, which represent the elements present on the surface. The analysis software was Genesis Spectrum, SEM Quant ZAF Version 3.60 (EDAX, Inc. NJ, USA).
3.3.7 **Tests performed by the manufacturer**

Data for these tests are presented in Appendix 6.

3.3.7.1 **Contact angle measurements**

All four surfaces had a contact angle of 90° (measured by the tensile drop method) and were considered to be slightly hydrophobic.

3.3.7.2 **Pre-sterilization testing**

Samples were taken (3 discs per reference number) from the lot prior to the beta-sterilization process. Each sample was transferred into a bottle containing 50ml NaCl with 0.1% peptone. The bottle was shaken for 10 minutes and then the solution was filtered. The filter was transferred to a TSA-plate and the plates were incubated at 32° C ± 2° C. The amount of colonies on the filter were counted.

3.3.7.3 **Endotoxin testing**

Endotoxin samples (3 discs per reference number) were taken from the lot after the beta sterilization process. Test samples were transferred into an endotoxin free tube and covered with 2ml sterile water and shaken for five minutes. Water was put in four different cups, then a solution of Control Standard Endotoxin (Endosafe) and LAL Reagent (Endosafe) water was added to two of them. The solutions were then inspected with Tecan/ Sunrise (microplate reader) equipment with the Endoscan V software.

3.3.7.4 **XPS**

The discs were analyzed on the surface with a XPS microscope (Quatum 2000 ESCA Scanning Microscope, Physical Electronics, USA). The monochromatic AlKα was used as the X-ray source. The beam was focused to 100 µm with the effect about 20W. Evaluation was accomplished using a matlab-based program, MULTIPAK (Physical Electronics, Lake Drive East, Chanhassen, MN). Three samples of the same batch were analyzed. The only XPS was performed on the Test discs 7874 TiOblast + HF due to the small production variation of F content.
3.3.8 Statistical Analysis

Statistical analyses was performed using the data-processing software SPSS (Version 21 for Mac; SPSS, Chicago, IL). The data was not found to be normally distributed, so the non-parametric analysis of variance, Kruskal-Wallis test was used to analyze the differences between the different surfaces regarding:

1. Number of Colony forming units/ ml
2. Biofilm surface areas (total, live and dead)
3. Biofilm volume (total, live and dead)
4. Biofilm thickness
5. Biofilm quantity and quality (live/dead) between the different layers of the biofilm

P-values < 0.05 were considered significant.
3.4 Results

3.4.1 Growth curves

Results showed that in 200 µl of mFUM media, *S. oralis* had a very short lag phase, followed by an extremely rapid growth rate in the exponential phase. Stationary phase was reached after 5 hours and continued for the rest of the 48 hours experiment (Figure 13).

![Growth curves for S. oralis. (media: mFUM, time = 48 h, absorbance λ = 600nm, n= 4) showing a rapid growth rate till 5h then stationary phase for the rest of the experiment](image)

3.4.2 Surface characterization of titanium discs

3.4.2.1 SEM of pristine surfaces

SEM images of discs 6905 and 7874 showed a relatively homogeneous roughened microstructure. Low-power images (800x) reveal similar micron-level topography. At a higher magnification (3000x and 6000x), 7874 appeared to have additional finer nano-sized irregularities interspersed between the larger micro-irregularities (Figure 14). Surfaces 7676 and 7873 showed a pronounced circular configuration of alternating plane and flattened areas consistent with a machining process. Surface imperfections or tags of rough areas were commonly observed. 7873 appeared to have some modification of this surface compared to 7676. These latter 2 surfaces could be classified as ‘smooth’ as compared to the other 2 ‘rough’ surfaces (Figure 15).
Figure 14 SEM of pristine rough surfaces 6905 (left) and 7874 (right)
a) magnification x800 b) magnification x3000 c) magnification x 6000.

TiOblast (left) The first clinically introduced moderately rough implant (Sa\(\approx\)1.0 \(\mu\)m)
Osseospeed (OS) surface (right) is a nanomodified version of the TiOblast (Sa \(\approx\) 1.5 \(\mu\)m).
Nanopores caused by acid treatment in the OS surface can be observed at 6000x
Figure 15 SEM of pristine smooth surfaces 7873 (left) and 7676 (right) at a) x800 magnification and b) x6000 magnification. Test discs show undulating regions, with ridges, valleys and occasional “flakes” produced from the machining process.
3.4.2.2 EDS OF PRISTINE SURFACES

EDS analysis revealed titanium as the predominant element in all four surfaces (Figures 16 and 17). Oxygen was only detected on the rough surfaces confirming the grit blasting procedure used with titanium dioxide particles to achieve a moderately rough surface (Figure 16). No fluoride was detected on any of the examined surfaces.

![Energy Dispersive X-ray Spectroscopy (EDS) analysis of rough surfaces](image)

**Figure 16** Elemental spectrum obtained by the EDS analysis of rough surfaces a) 6905 b) 7874 showing peaks caused by X-rays given off as electrons return to the K electron shell. Note the large peak for Titanium, which was the predominant element. Oxygen was also detected.
Figure 17 Elemental spectrum obtained by the EDS analysis of smooth surfaces a) 7873 b) 7676. Titanium is the predominant element. No Oxygen was detected.
3.4.3 Biofilm Formation on the Different Surfaces after 96 Hours

3.4.3.1 SEM of surfaces with biofilm

SEM images in (figures 18 and 19) show the adhesion of *S. oralis* to the different titanium surfaces. A biofilm made up of intertwined cocci arranged in short and long chains characteristic of streptococcal species could clearly be seen. However no differences in colonization patterns could be distinguished amongst the four surfaces.

![SEM images](image-url)

*Figure 18* Representative SEM micrographs of smooth surfaces 7873 (left) and 7676 (right) with *S. oralis* 96h biofilm a) x800 magnification and b) x6000 magnification showing confluent layers of cocci on the surfaces
Figure 19 Representative SEM micrographs of rough surfaces 6905 (left) and 7874 (right) with 96h *S. oralis* biofilm showing confluent layers of cocci a) 800 x b) 3000x c) 6000 x
3.4.3.2 Bacterial Counts

CFU counts obtained from harvesting and culturing the adherent biofilms are summarized in Figure 20 and in table 19. There was no significant difference between the numbers of colonies counted for any of the surfaces tested.

Figure 20 The number of colony forming units after 96 h in vitro biofilm growth (n=10). Medians and 25/75 percentiles are shown. Kruskal Wallis ANOVA analysis failed to reveal any significant differences (P>0.05) between the surfaces.

<table>
<thead>
<tr>
<th>Surface</th>
<th>6905</th>
<th>7874</th>
<th>7873</th>
<th>7676</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Bacterial Count (x10^6)</td>
<td>46 (6–84)</td>
<td>44 (11–72)</td>
<td>57 (6–71)</td>
<td>41 (19–73)</td>
</tr>
</tbody>
</table>

p = 0.998*

Table 19 Total Bacterial Counts (medians and 25/75 percentiles) of 96 h biofilm (n=10)*Kruskal Wallis analysis of variance
3.4.3.3 CLSM Analyses

Examples of the CLSM microscopic images are shown in Figure 21. *S. oralis* species adhered readily to all surfaces. Using the STYO® 9 and propidium iodide enabled visual differentiation of the live and dead bacteria. The software bioimage_L processed the confocal images using in situ colour segmentation based on the colour additive theory, which classifies each pixel of the image into predefined colour classes. The colour to be segmented is identified by green, red, and blue codes which are subtracted from each pixel in the image. The green and red colour classes formed the basic units of analysis in this instance.
Figure 21 CLSM evaluation of 96h biofilm. Chains of *S. oralis* are apparent. Live green (STYO®9 stain) bacteria, dead red (propidium iodide stain) bacteria and overlaid image showing both. Left panel 2x magnification. Right panel 6x magnification. Area 45032µm²
Although rougher surfaces, 6905 and 7874 theoretically provided a greater surface area for bacterial adhesion and protection for the bacteria against shear removal forces, no significant differences could be detected in surface areas covered by cells on any of the surfaces (Figure 22, Table 20).

**Figure 22** Surface area covered with *S. oralis* after 96 h *in vitro* biofilm growth (n=10). Medians and 25/75 percentiles are shown. Kruskal Wallis ANOVA analysis failed to reveal any significant differences (P>0.05) between the surfaces.

<table>
<thead>
<tr>
<th>Disc Type</th>
<th>6905</th>
<th>7874</th>
<th>7873</th>
<th>7676</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total biofilm area (µm²x10^5)</td>
<td>7.5 (6.1-10.7)</td>
<td>6.9 (5.3-7.4)</td>
<td>5.1 (3.8-9.9)</td>
<td>8.5 (5.7-10.8)</td>
<td>0.207</td>
</tr>
<tr>
<td>Live cell area (µm²x10^5)</td>
<td>5.8 (5.0-7.6)</td>
<td>6.6 (3.9-6.8)</td>
<td>4.4 (3.1-9.4)</td>
<td>6.9 (4.8-9.1)</td>
<td>0.425</td>
</tr>
<tr>
<td>Dead cell area (µm²x10^5)</td>
<td>0.7 (0.02-3.6)</td>
<td>0.8 (0.03-1.3)</td>
<td>0.8 (0.05-1.0)</td>
<td>0.6 (0.2-2.1)</td>
<td>0.838</td>
</tr>
</tbody>
</table>

Table 20 Biofilm analysis showing medians (and interquartile range between parentheses) of biofilm surface area (total, live and dead). (n=10)*Kruskal Wallis analysis of variance
Furthermore, the biofilm volume produced was also comparable between the four surfaces (Figures 23, Table 21).

![Box plot showing biofilm volume formation on different surfaces](image)

**Figure 23** Biofilm volume formed on surfaces after 96h (n=10). Medians and 25/75 percentiles are shown. Kruskal Wallis analysis failed to reveal any significant differences (p>0.05) between the surfaces.

<table>
<thead>
<tr>
<th>Disc Type</th>
<th>6905</th>
<th>7874</th>
<th>7873</th>
<th>7676</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total biofilm volume (µm³x10⁶)</td>
<td>15.1 (12.1-21.3)</td>
<td>13.8 (10.6-14.7)</td>
<td>10.4 (7.5-19.9)</td>
<td>14.3 (10.9-20.0)</td>
<td>0.398</td>
</tr>
<tr>
<td>Live cell volume (µm³x10⁶)</td>
<td>11.7 (9.9-15.1)</td>
<td>13.3 (7.8-13.6)</td>
<td>8.8 (6.2-18.8)</td>
<td>11.7 (9.1-18.0)</td>
<td>0.704</td>
</tr>
<tr>
<td>Dead cell volume (µm³x10⁶)</td>
<td>1.4 (0.5-7.2)</td>
<td>1.5 (0.5-2.7)</td>
<td>0.8 (0.1-2.1)</td>
<td>1.2 (0.3-4.2)</td>
<td>0.716</td>
</tr>
</tbody>
</table>

**Table 21** Biofilm volume (total, live and dead). (n=10)*Kruskal Wallis analysis of variance
The percentage of the surface area covered by biofilm was between 78-79% for surfaces 6905, 7874 and 7676, whilst surface 7873 tended to have less surface coverage (59%). The difference did not reach statistical significance (Figure 24, Table 22).

**Figure 24** Percentage of biofilm coverage on the different surfaces after 96 h incubation with *S. oralis* (n=10). Medians and 25/75 percentiles of biofilm coverage are shown. Kruskal Wallis analysis failed to reveal any significant differences (*p*>0.05)

<table>
<thead>
<tr>
<th>Disc Type</th>
<th>Percentage (%) of Surface covered</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6905</td>
<td>78.6 64.5 - 89.6</td>
<td></td>
</tr>
<tr>
<td>7874</td>
<td>78.2 61.8 - 81.7</td>
<td></td>
</tr>
<tr>
<td>7873</td>
<td>59.2 50.6 - 90.6</td>
<td></td>
</tr>
<tr>
<td>7676</td>
<td>79.1 67.2 - 93.0</td>
<td>0.351</td>
</tr>
</tbody>
</table>

**Table 22** Percentage of disc surface covered by cells (n=10)*Kruskal Wallis analysis of variance
The mean height/thickness of the developed biofilm was 8.3 ± 0.5 µm (Figure 25)

![Mean height graph](image)

**Figure 25** Mean height of *S. oralis* biofilm after 96h on titanium surfaces (n=10). Mean height was lowest on the nanorough surface with fluoride (7874).

Despite previous analyses showing a tendency for the nanorough surface with fluoride 7874 to consistently have lower bacterial adhesion, biofilm volume and surface coverage compared to both the moderately rough 6905 surface and the smooth 7676 surface, this was not statistically significant.
The proportion of live/dead cells was approximately 90% live to 10% dead cells on all four surfaces (Figure 26).

**Figure 26** a) Surface area and b) volume covered by live and dead *S. oralis* bacteria after 96h on different titanium surfaces (medians and interquartile range) n=10
Finally, the biofilm nearest the disc surface (at 2 and 4 µm) was compared to that furthest away from it (at 18 and 20 µm) to determine whether physiochemical surface differences had an effect on biofilm characteristics. Assessment of the biofilm did not reveal any significant differences (Table 23). An illustration of the mean biofilm mass at the different z-levels can be seen in Figure 27.

<table>
<thead>
<tr>
<th>Disc Type</th>
<th>6905 (479-2949)</th>
<th>7874 (175-1361)</th>
<th>7873 (191-1900)</th>
<th>7676 (385-3311)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total at 2 µm</td>
<td>1042</td>
<td>343</td>
<td>462</td>
<td>1415</td>
<td>0.220</td>
</tr>
<tr>
<td>Live at 2 µm</td>
<td>609</td>
<td>81</td>
<td>184</td>
<td>335</td>
<td>0.381</td>
</tr>
<tr>
<td>Dead at 2 µm</td>
<td>608</td>
<td>206</td>
<td>116</td>
<td>541</td>
<td>0.555</td>
</tr>
<tr>
<td>Total at 4 µm</td>
<td>3046</td>
<td>1322</td>
<td>1361</td>
<td>2395</td>
<td>0.185</td>
</tr>
<tr>
<td>Live at 4 µm</td>
<td>1170</td>
<td>363</td>
<td>848</td>
<td>1257</td>
<td>0.371</td>
</tr>
<tr>
<td>Dead at 4 µm</td>
<td>1251</td>
<td>621</td>
<td>279</td>
<td>1208</td>
<td>0.699</td>
</tr>
<tr>
<td>Total at 18 µm</td>
<td>159008</td>
<td>169247</td>
<td>128623</td>
<td>175924</td>
<td>0.309</td>
</tr>
<tr>
<td>Live at 18 µm</td>
<td>139334</td>
<td>157786</td>
<td>107024</td>
<td>158638</td>
<td>0.403</td>
</tr>
<tr>
<td>Dead at 18 µm</td>
<td>10989</td>
<td>10200</td>
<td>9575</td>
<td>6465</td>
<td>0.799</td>
</tr>
<tr>
<td>Total at 20 µm</td>
<td>138636</td>
<td>140680</td>
<td>113222</td>
<td>155101</td>
<td>0.684</td>
</tr>
<tr>
<td>Live at 20 µm</td>
<td>128524</td>
<td>119131</td>
<td>102693</td>
<td>145503</td>
<td>0.794</td>
</tr>
<tr>
<td>Dead at 20 µm</td>
<td>3540</td>
<td>3209</td>
<td>1035</td>
<td>2282</td>
<td>0.824</td>
</tr>
</tbody>
</table>

Table 23 Medians (inter-quartile range) of biofilm surface area (µm²) at different Z-levels (2 µm, 4 µm, 18 µm and 20 µm) from the disc surface *Kruskal Wallis analysis of variance
Figure 27 Distribution of *S. oralis* biofilms at different z-levels (n=10) after 96h incubation

(6905)

(7874)
3.5 DISCUSSION

Dental biofilms have been associated with the initiation and progression of caries and periodontal disease (Liljemark et al., 1997). The control of these biofilms is fundamental for maintenance of a healthy mouth and the prevention of gingivitis and periodontitis. Similarly there is considerable evidence that supports the cause and effect relationship between biofilm accumulation and implant related disease.

Biofilms have been shown to be resistant to standard antibiotic therapy and the development of new implant surfaces that aim to optimize bone healing, but reduce microbial colonization may provide a potentially more promising approach (Palmquist et al., 2010b)

Some research has shown that titanium has inherent antibacterial properties indicating that 250-500 ppm of pure titanium and 125 ppm of titanium alloy would inhibit the growth of some oral microorganisms in vitro. However these findings have limited clinical application since it is unlikely that such high concentrations exist in the intra-oral environment (Berry et al., 1992)

In our current study, we used a single species model to investigate in vitro microbial adhesion to four different titanium surfaces and intended to correlate the findings to specific surface characteristics (roughness and chemical modification). The aim was to provide an insight to which surface characteristics could minimize biofilm formation and reduce the risk of peri-implant disease.

There were no significant differences in biofilm formation on any of the surfaces after 96 hours regardless of surface roughness or chemical composition. This is in contrast to the opinion that a roughness threshold value of 0.2 μm will affect colonization (Quirynen et al., 1996a).

It has been proposed that rougher surfaces provide more microniches that may provide a more favourable, protective environment that facilitates bacterial interactions. Similarly, fluoride has been proposed to reduce adhesion of oral bacteria (Loskill et al., 2013, Yoshinari et al., 2001). Although, the fluoride treated discs (7874) tended to exhibit less bacterial adhesion patterns, this did not reach statistical significance.
It appears that once initial colonization and early biofilm formation is established the surface has little effect thereafter. This is in line with other studies conducted on titanium by Frojd et al. (Frojd et al., 2011a, Frojd et al., 2011b) which showed that differences in surface roughness may have an effect after 2 hours, but after 14 hours this effect was not sustained suggesting that the influence of the surface characteristics on adhesion was compensated for by biofilm development. The same observations were also seen on different implant and dental surfaces (Siegrist 1991, Leonhardt et al., 1995). Dezelic et al. showed that surface roughness may influence initial biofilm adherence after 15 min, but differences disappeared following growth and maturation after 15 hours (Dezelic et al., 2009). Furthermore, Nakazoto et al. observed that surface roughness of materials may affect initial bacterial adherence at 4 hours, but at 48h, there was no difference in bacterial adhesion among any of the materials tested (Nakazato et al., 1989). Another study showed that the influence of surface roughness was still apparent after 3 days, but disappeared after 5 days (Al-Ahmad et al., 2010). Following on from this, it may be surmised that moderately roughened implant materials could possibly increase initial bacterial adhesion but had little effect as the biofilm developed. Nonetheless, a recent study has observed that even at 20 minutes biofilms were similar on materials that had different surface roughness (Schmidlin et al., 2013).
CHAPTER 4  THE EFFECT OF VARIOUS DECONTAMINATION METHODS ON RE-ESTABLISHING BIOCOMPATIBILITY OF CONTAMINATED IMPLANT SURFACES (STUDY 3)
4.1 INTRODUCTION

4.1.1 THE THERAPY OF PERI-IMPLANTITIS

Evidence is still limited as to which therapeutic strategies are the most efficacious for the treatment of peri-implantitis. The vast heterogeneity of studies, which have used empirical combinations of different decontamination methods, and the variability of implant surfaces being treated, make it is difficult to establish a single protocol for peri-implantitis treatment, but this does not suggest that the currently implemented treatment modalities may not provide beneficial outcomes in clinical practice (Kotsovilis et al., 2008).

Favourable treatment outcomes have been correlated with non-smoking and late disease development (Charalampakis et al., 2011) and the following clinical guidelines for therapy are recommended (Heitz-Mayfield and Mombelli, 2014):

- Oral hygiene instruction and smoking cessation advice.

- Assessment of the prosthesis for access for plaque control and if required, removal and adjustment of the prosthesis.

- Non-surgical therapy should be attempted initially, with or without antimicrobials, before resorting to surgical techniques (Lindhe et al., 2008, Renvert et al., 2008).

- Surgical access to allow thorough cleaning of the decontaminated implant surface. This may involve resective or regenerative treatment and also the modification of the implant surface topography.

- Continuous maintenance, including oral hygiene instructions and supramucosal biofilm removal.

Detailed algorithms set by the ADI for the non-surgical and surgical therapeutic approaches are described in Appendix 1.
4.1.2 Decontamination of the Implant Surface

Because peri-implantitis is initiated by microorganisms, the primary objective of treatment, is the elimination of infection through removal of the biofilm and decontamination of the implant surface rendering the surface biocompatible for re-osseointegration (Renvert and Giovannoli, 2012).

A promising method of achieving this seems to be the use of air abrasion (Meyle, 2012). Two interesting powders that can be used in APDs for the decontamination of dental implants are glycine and BAG. The former is a soft alternative to the conventional sodium bicarbonate powder. It has been developed recently in order to be used subgingivally without inducing substantial soft and hard tissue damage (Flemmig et al., 2007, Petersilka et al., 2008). The latter, BAG possesses osteoinductive, osteoconductive and antimicrobial properties (Hench, 2006, Stoor et al., 1998) and has not been tested before for the treatment of peri-implantitis. It would seem reasonable to presume that BAG, used in an air-polishing device would be able to effectively disrupt the plaque biofilm on dental implants and at the same time impart a level of bioactivity to the surfaces. An anticipated beneficial effect of BAG particles being retained on the implant surface would be to enhance reosseointegration and prevent the formation of an intervening fibrous tissue.

*In vitro* studies give an important insight to the expected biocompatibility results *in vivo*. Generally, two types of cells are used for *in vitro* studies, these are primary cells and cell lines. Primary cells are advantageous for studies of bone cell metabolism and differentiation because they retain a normal phenotype since no dedifferentiation has taken place (Aubin et al., 1995, Di Silvio and Gurav, 2001). A few *in vitro* studies (Augthun et al., 1998, Kreisler et al., 2005, Parham et al., 1989, Schwarz et al., 2009a, Shibli et al., 2003) have suggested that the biocompatibility of titanium surface following air-polishing to be high. However, only one (Schwarz et al., 2009a) has used glycine and none have used BAG. Furthermore, all the aforementioned studies used cells other than those of human origin or used cells that do not represent dental implant targets (i.e. they used human osteosarcoma cells, mouse preosteoblast cell lines or fibroblasts). In an ideal *in vitro* approach, biomaterials should also match the cell populations typical of the implant site. In the case of dental implants, these are mainly alveolar human osteoblasts, as it has been
shown that osteoblasts taken from different sites of the body may exhibit different proliferation rates and phenotypic expression.

Therefore, the aim of this *in vitro* study was to assess using primary alveolar human osteoblasts (HOB), how decontamination procedures with air polishing devices using 2 different powders, one ‘soft’ (glycine) and one ‘bioactive’ (BAG) would re-establish biocompatibility of previously contaminated, commercially-available implant surfaces and how this compared to decontamination using mechanical debridement and non-contaminated, sterile surfaces.
4.2 STUDY 3 AIMS AND OBJECTIVES

Aims

Investigate and compare the effect of different decontamination methods on dental implant surfaces in recreating a biocompatible surface to which cell reattachment can occur.

Objectives

Biocompatibility and effect of decontaminants on the titanium surfaces will be assessed using human alveolar osteoblast cells. This will be measured using:

1- MTT to test cytotoxicity

2- Alamar blue to test cell proliferation

3- Alkaline Phosphatase activity as a marker of differentiation

4- SEM/ EDS for topographical and chemical characterization of surfaces after decontamination
4.3 Materials and Methods

4.3.1 Titanium Surfaces and Biofilms

Biofilms were grown on the implant discs as described in the previous chapter. Immediately after the biofilm experiment was complete, the discs were allocated to the specific treatment procedures. Two discs of each implant surface per treatment method were analyzed in addition to two control discs of each surface which were non-contaminated and untreated.

4.3.2 Treatment Procedures

The following treatments were compared:

4.3.2.1 Mechanical Debridement

Disposable scalpels (Swann-Morton, Sheffield, UK) with a number 11 blade were used as a sterile, reproducible and simple alternative to a curette for scaling the disc. A new blade was used for each disc to avoid bacterial contamination from one disc to the other.

Each disc was scaled for 20 seconds (from top to bottom ≈10 times then ≈10 times from left to right). The blade was placed at a 90 degrees angle to the disc.

According to Duarte et al. (Duarte et al., 2009b), 6 strokes with a curette simulate approximately 1 clinical visit for implant decontamination. Thus 20 strokes should simulate around 4 clinical visits.

4.3.2.2 Air-Powder Abrasive/Polishing System

Two different types of powders were used:

a) Glycine (Airflow® subgingival PERIO powder, EMS Electro Medical Systems, UK)

b) Bioactive Glass (BAG) 45S5 – calcium sodium phosphosilicate (Sylc®, Denfotex Research Ltd, UK)
4.3.3 Hand-piece Settings

An AIR-FLOW® handy 2+ hand piece (EMS Electro Medical Systems, UK) was mounted on an adjustable stand and air-powder blasting was performed in a standardized manner according to the following parameters:

Pressure: 4.5 bar according to manufacturer recommendation.

Nozzle distance: 5 mm from the centre of the disc

Nozzle angle: 90˚ to the surface

Duration: 5 seconds static and 15 seconds rotation (total 20 seconds).

Nozzle distance and angulation were deduced from previous studies (Kreisler et al., 2005, Augthun et al., 1998) that used the same parameters.

The movement and duration parameters were determined after conducting the following pilot study:

Discs were painted with nail polish and blasted with the hand piece nozzle at

a) 5 mm for 5 seconds static, then 15 seconds rotation

b) 10 mm static only for 15 seconds

c) 5 mm static only for 15 seconds

d) 5 mm rotation only for 15 seconds

It was concluded that the best results were achieved using the combination of static and rotational movements (Figure 28).
A vortex mixer (Whirlmixer™ Fisons SGP-202-010J, Fischer Scientific UK) was used to act as a rotary mechanical stage. The potentiometer on the underside of the unit and the dial control on the front were adjusted to the lowest setting to provide the slowest rotational speed motion.

A putty – mount assembly was used to mount the discs in the vortex mixer. Initially the disc was placed in a suitable indentation created in the putty. However there were 2 problems that arose during piloting of the method. First, was it was difficult to put the disc in place without touching the top and disturbing the biofilm. The second problem was the air pressure occasionally causing the disc to fly out. To overcome this, the mount supplied with the implant disc packaging was embedded in the putty material (Aquasil Soft Putty, Dentsply, UK) and the discs were screwed on to it. This provided a firm and secure attachment to the discs and enabled handling the discs without having to touch the treated top surface.
Finally, the AIR-FLOW® handy 2+ reservoir was filled to the same level (maximum line) before every set of discs (a set of disc denotes 4 discs, 1 of each type) was blasted. This ensured that the reservoir was never less than half-way full at any instance.

4.3.4 Sterilization of the Discs

Sterilization of test samples was performed prior to cell culture. Two methods were considered, autoclaving and gamma radiation. According to previous studies, the chemical composition of the titanium implant surfaces that were sterilized by Gamma radiation showed a similar distribution of elemental concentration as control surfaces while the surfaces sterilized by autoclaving showed a higher percentage of Carbon. Autoclaving also induced an increase in water contact angle (Park et al., 2012, Drake et al., 1999) and resulted in a decreased surface roughness due to the deposition of steam-borne contaminants (Park et al., 2012). When re-exposed to S. sanguinis, surfaces that were autoclaved showed an increased bacterial colonization (Drake et al., 1999). Taken together, this data suggested that gamma radiation was the better method suited to sterilize the discs for the objectives of this study, hence discs were gamma radiated after the treatment procedures and before the cell culture assays.

4.3.5 Cell Cultures

Primary human osteoblast (HOB) cells derived from the alveolar bone of subjects undergoing third molar extraction were used at passages 10-15. Ethics and patient consent was obtained and approved (05/Q1803/85).

All cell culture procedures were performed under aseptic conditions in a Class 2 safety cabinet (Astec-Microflow Class II ABS, Bioquell UK) and incubation was carried out in a humidified atmosphere with CO₂ at 37°C in 5% CO₂ (Heracell™ 150i, Thermo Scientific, UK) unless stated otherwise.
4.3.5.1 Resuscitation of Cryopreserved cells

HOB cells were resuscitated for expansion for cellular studies. Cells were thawed by warming them to room temperature and adding 1 ml of HOB culture media drop wise in order to avoid osmotic shock to the cells. The HOB culture media was made up of Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma, UK), supplemented with 10% foetal calf serum (FCS, Sigma UK), 1% minimal non-essential amino acids (Sigma UK), 200 mM L-glutamine (Sigma UK), 1M HEPES (Sigma UK), penicillin-streptomycin 100x (Sigma UK) and L- ascorbic acid (150 µg/ml) (Sigma UK). The cells were then transferred to a sterile universal tube and made up into 10 ml with the media.

This mixture was centrifuged at 2000 rpm for 5 minutes (Eppendorf Centrifuge 5702, Eppendorf, UK). Following centrifugation, excess media was discarded and the remaining cell pellet was resuspended in fresh HOB media and homogenized then transferred to one or two 25 cm$^3$ culture flasks according to the size of the pellet. The flasks were incubated in a humidified atmosphere with CO$_2$ at 37°C in 5% CO$_2$ (Heracell™ 150i, Thermo Scientific, UK).

4.3.5.2 Subculturing

Cells were checked microscopically daily to ensure they were healthy and growing as expected and the media was changed every 3 days. Cells were subcultured when they were 80% - 90% confluent.

Confluent cells were harvested by trypsinization. This involved removing the spent medium from the flasks, rinsing the cells with phosphate buffered saline (PBS, Sigma UK), adding 1ml of diluted (1x) trypsin (Sigma UK) and incubating for 5 minutes.

Detached cells were observed under an inverted light microscope (Primo Vert, Carl Zeiss, UK) and dislodged by gentle tapping or scraping with a cell scraper if necessary. 10 ml of HOB media was added to inactivate the trypsin and stop the enzymatic reaction. The cell suspension was removed to sterile universal tubes, centrifuged at 2000 rpm for 5 minutes, the supernatant was then discarded and the pellet re-suspended in fresh media and cells were either subcultured in a 1:2 split ratio or counted and used for the experiments as described in the following section.
4.3.5.3 CELL COUNTING AND CELL VIABILITY

Cell counting was conducted before cells were seeded on the implant discs using Trypan Blue, 0.4% vitality dye (Life Technologies, UK). A Bio-Rad TC10 automated cell counter (Bio-Rad, UK) was used to count the total and live cells. This was done in duplicates and the mean number of live cells recorded.

4.3.5.4 SEEDING CELLS ON IMPLANT DISCS (FOR ALAMAR BLUE AND ALKALINE PHOSPHATASE ASSAYS)

After gamma radiation, the discs were placed in 96 well plates with treated surfaces for cell culture (Nunc™ MicroWell™ 96-Well Microplates, Thermo Scientific UK). 200µl of HOB cell suspension containing 1 x 10⁴ cells was pipetted onto each disc and the plate was incubated. Tissue culture plastic was used as the negative (non-toxic) control.

4.3.6 CYTOTOXICITY TESTING BY MTT

MTT was performed to measure cell metabolic activity. Material toxicity was assessed indirectly by exposing cells to media containing leachable components from the decontaminated discs (eluants).

This colourimetric assay is based on the reduction of tetrazolium salt (MTT) by mitochondrial dehydrogenases. During incubation, the redox system reduces this yellow salt to an intracellular insoluble bluish - purple formazan. The amount of purple formed should be proportional to the number of viable cells as this enzyme is only present in intact, living cells.

Discs were placed in bijous containing 5 ml HOB media under sterile conditions. The bijous were rotated for 24 and 72 hours on a DENLEY Spiramix. The eluants were collected and transferred to clean bijou vials and stored at – 20° C till they were ready to be used for the MTT assay. In the meantime, 96 well plates were seeded with cells at a density of 1 x 10⁴ cells/well and incubated for 24 hours with 100 µl of media to allow the cells to acclimatize. After that, the media was replaced with 100 µl of the eluants and incubated for either 24 or 72 hours “exposure time”.

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An MTT solution was prepared by dissolving 5 mg of 3-(4,5) dimethythiazol-2-yl-2,5 diphenyl tetrazolium bromide in 1 ml of sterile phosphate buffered saline. This was added to 10 ml of HOB medium without ascorbic acid.

At the selected exposure times, the elution media was discarded and replaced with 100 µl of MTT solution per well. Negative (non toxic) and positive (toxic) controls were prepared comprising media only and 10% alcohol diluted in media respectively. The plates were incubated for a further 4 hours and then the solution was replaced with 100µl of DMSO (Sigma) per well and shaken for 5 minutes to ensure dissolution of formazan crystals. Absorbance was measured using a spectrometer (Dynex) at 570 nm test and 620 nm reference wavelengths.

4.3.7 Osteoblast Proliferation Assay – alamarBlue®

The alamarBlue® is a fluorometric/ colourimetric assay which uses the reducing power of viable cells to measure the proliferation of cells. When cells are metabolically active, they maintain a reducing environment within the cytosol of the cell. Resazurin (REDOX indicator), the active ingredient of alamarBlue® reagent, is a non-toxic, cell permeable compound that is blue in color and virtually non-fluorescent. Upon entering cells, resazurin is reduced via an oxidation-reduction reaction to resorufin, a compound that is reddish-pink in color and highly fluorescent. Viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and colour of the media. The reduction of alamarBlue® is relative to cell growth allowing quantitative analysis of cell metabolic activity. The lighter the pink colour of the alamarBlue®, the higher the mitochondrial activity, and hence, the cell proliferation.

Advantages of this assay is that it is relatively simple, highly sensitive, quantitative and does not necessitate killing cells i.e. not an end point assay, thus allows continuation of culture or further assays to be performed if necessary.

Cell proliferation was evaluated at days 3, 7, 14, 21 and 28. At each time point, the culture media was replaced with 250 µl of alamarBlue® solution (10% v/v alamarBlue® in phenol red-free medium) and the plate returned to the incubator for 4 hours. After 4 hours, 100 µl was removed to a 96 well plate. The solution was transferred to a 96 well plate in duplicates.
(100 µl per well) and the absorbance was measured at a wavelength 570 nm (reference wavelength 620 nm) wavelengths using a spectrophotometric plate reader (DYNEX Opsys technologies reader). Excess alamarBlue® was removed and the samples were washed 3 times with PBS then 200 µl HOB media was added to each well for further incubation to the next time point. Media was changed twice a week.

4.3.8 Osteoblast differentiation assay - Alkaline Phosphatase

Proliferation is followed by matrix maturation, during which the matrix is prepared for the deposition of the mineral phase of bone by alkaline phosphatase (ALP), an enzyme associated with mineralization within bone and cartilage and is used as a marker of osteoblast differentiation (Figure 29).

![Diagram of osteoblast differentiation](image)

*Figure 29 Proliferation of osteoblasts is shown to decrease as the cells terminally differentiate into matrix production. Taken from (Burling, 2005), adapted from (Stein et al., 1996)*
ALP production in HOB cells was determined by measuring the ALP activity in cell lysates obtained from freeze/thaw procedure. At the required time-point, the supernatant HOB media was removed from the samples (including controls) and 200 µl of sterile UHQ distilled water was added to each sample. The samples were freeze-thawed 3 times by placing the well plate at –80°C for 20 minutes followed by thawing at 37°C for 15 minutes. In between each cycle, the suspensions were thoroughly mixed to ensure cell dispersion and to obtain a homogenous cell lysate. The lysates were collected and stored at –80°C until needed.

50 µl of cell lysates were aliquoted in 96 well plates, to which 50 µl of ALP substrate reagent (p-nitrophenol phosphate, magnesium chloride hexahydrate, and triton X-100 in 0.1 M glycine, all Sigma) was added. A standard curve was obtained by serially diluting p-nitrophenol (Sigma) in 0.1 glycine (Sigma) at pH 10.3 from 100 to 1.563. 100 µl of each dilution was pipetted into the appropriate wells. Plates were shaken for 2 minutes on a Titertek plate shaker and then incubated at 37°C for 20 minutes.

In this reaction, the substrate p-nitrophenylphosphate is hydrolyzed into phosphate and p-nitrophenol in the presence of the enzyme alkaline phosphatase. The product is yellow in colour and the intensity is proportional to the concentration of alkaline phosphatase.

After 20 minutes, the plates were placed in a plate reader (Dynatech) and absorbance was measured at wavelength 405 nm (reference wavelength 620 nm).
4.3.9 Topographical and chemical characterization of the decontaminated surfaces using SEM and EDS.

4.3.9.1 Preparation of surfaces for SEM and EDS

For descriptive analysis of titanium surface changes after the samples were decontaminated, 24 discs (2 discs of each surface subjected to each type of treatment i.e. 2 x 4 surfaces x 3 treatments) were evaluated using SEM and images were acquired at 3000x and 6000x magnification. EDS operating at 12 kV was also carried out on the discs that were decontaminated using air-powder blasting to compare the chemical composition of the surface with that of the pristine surfaces Figure 16.

The same protocol used for the pristine surfaces was used for the decontaminated surfaces (chapter 3, page 127). In addition, the samples were sputter coated according to the decontamination method used as follows:

a) Mechanically cleaned samples were gold coated, as described previously because we were not intending to carry out any EDS analysis on them.

b) Samples decontaminated with bioactive glass blasting were carbon coated using an Emitech K250 sputter coater.

c) Samples cleaned with glycine powder blasting were gold coated after EDS analysis was carried out with no coating.
4.3.10 Data Analysis

The alamarBlue and alkaline phosphatase experiments were repeated three times while the MTT and scanning electron microscopy experiments were performed only once. Two measurements were taken from each disc in the alamarBlue and alkaline phosphatase assays, and three measurements per disc for the MTT assay.

Titanium discs were allocated to assays as described in Table 24.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Total</th>
<th>SEM</th>
<th>MTT</th>
<th>AB</th>
<th>ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td>6905</td>
<td>110</td>
<td>6</td>
<td>8</td>
<td>24</td>
<td>72</td>
</tr>
<tr>
<td>7874</td>
<td>110</td>
<td>6</td>
<td>8</td>
<td>24</td>
<td>72</td>
</tr>
<tr>
<td>7873</td>
<td>110</td>
<td>6</td>
<td>8</td>
<td>24</td>
<td>72</td>
</tr>
<tr>
<td>7676</td>
<td>110</td>
<td>6</td>
<td>8</td>
<td>24</td>
<td>72</td>
</tr>
</tbody>
</table>

Table 24 Allocation of titanium discs to different assays (SEM - scanning electron microscopy, MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AB - alamarBlue; ALP - Alkaline phosphatase)

Data were expressed as means ± standard deviation and one way ANOVA followed by post hoc testing using Scheffe’s correction for multiple comparisons (p < 0.05) to detect if there were statistically significant differences between the different types of treatments and osteoblast proliferation and/or differentiation.

A schematic overview of the experiment is illustrated in Figure 30.
Figure 30 Schematic diagram showing the workflow of the tissue culture assays. Discs were decontaminated at day ‘0’ then sent for gamma radiation/ SEM. As soon as they were back, cells were plated at the desired concentration on the discs and assayed after 1, 2 days of culture (MTT); 3, 7, 14, 21, 28 days of culture (AB); 7, 14, 21 days of culture (ALP). (AB, alamarBlue; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; ALP, alkaline phosphatase)
4.4 RESULTS

4.4.1 CELLULAR RESPONSE TO DECONTAMINATED TITANIUM SURFACES

4.4.1.1 CYTOTOXICITY AS ASSESSED BY MTT

Samples were eluted for 24 and 72 hours to detect if any leached products could affect cell behavior. The cytotoxicity of samples and metabolic activity of the cells were assessed by exposure to MTT for 24 and 72 hours. The results of these indirect test elution studies are shown in Figure 31 and 32.

Eluants taken after 24 hours from decontaminated and control implant surfaces showed slightly less absorbance values compared to the negative ‘media only’ control (figure 31). There was an increase in absorbance values after 72 hours exposure for all the materials in general, and were comparable to the negative control indicating that there were no cytotoxic detrimental effects to the cells (figure 32). Also, the elutions obtained after 72 hours from the rougher surfaces (7874 and 6905), showed greater absorbance irrespective of incubation (exposure time) or treatment method (figure 32). One way ANOVA test demonstrated that there were no significant differences (p > 0.05) found between any of the decontamination methods used, while a significant difference (p < 0.001) was observed between the positive toxic control and the eluants from the materials at each test period.
Figure 31 MTT assay showing mitochondrial activity of HOB cells after 24 h and 72 h exposure to the 24 h eluants of different implant surfaces after being subjected to different decontamination methods surface. Longer exposure time in elutions was accompanied by increased viability. (n=2)
Figure 32: Results of MTT assay showing HOB cell response after 24 h and 72 h exposure to the 72h eluants of each surface. There was an increase in absorbance values after 72 h exposure for all the materials in general (n=2).
4.4.1.2 **Direct contact proliferation assay (AlamarBlue)**

Results were obtained at day 3, day 7, day 14, day 21 and day 28. Proliferation activity of the cells on the titanium surfaces in the four groups is presented in Figure 33. Proliferation pattern was more or less similar for all surfaces and treatment methods. There was a progressive increase in cell number with proliferation peaking at days 14 and 28. A drop in proliferation rate was observed at day 21 compared to day 14. Tissue culture plastic was used as a negative control and showed the same pattern of proliferation for the osteoblasts. Each experiment was conducted in duplicates obtaining 2 readings from each disc. This experiment was repeated three times (n = 6).
Figure 33 Proliferation of HOB cells as determined by the alamarBlue assay showing each surface and variation with time after being subjected to various decontamination methods. Control discs were non-contaminated and non-treated. The values are means ± standard deviation (n=6). Significantly lower proliferation rate on surface 7873 at days 7, 14 and 21 after air blasting, with BAG and glycine, compared to the mechanical debridement (p<0.05). Also on surface 7676 at day 14 after glycine blasting compared to mechanically debridement (p=0.04).
A positive proliferative response of HOB cells was observed with all samples and treatments, proving re-establishment of biocompatibility comparable to control specimens. However, there was significantly lower proliferation rate on surface 7676 at day 14 after glycine blasting compared to those that were mechanically debrided (p=0.04). For surfaces 7873, significant differences were observed at days 7, 14 and 21 in which air-blasted surfaces, with BAG and glycine, showed less proliferation compared to the scaled surfaces (p<0.05). Differences are summarized in Table 25.

<table>
<thead>
<tr>
<th>Day</th>
<th>Surface</th>
<th>Decontamination method</th>
<th>Less proliferation than decontamination by</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>7676</td>
<td>Glycine</td>
<td>Scaler</td>
<td>0.04</td>
</tr>
<tr>
<td>7</td>
<td>7873</td>
<td>BAG</td>
<td>Control</td>
<td>0.02</td>
</tr>
<tr>
<td>14</td>
<td>7873</td>
<td>Glycine</td>
<td>Scaler Control</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>0.03</td>
</tr>
<tr>
<td>21</td>
<td>7873</td>
<td>BAG</td>
<td>Scaler</td>
<td>0.004</td>
</tr>
<tr>
<td>21</td>
<td>7873</td>
<td>Glycine</td>
<td>Scaler</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Table 25 Time points, surfaces and decontamination methods at which significant differences in HOB cell proliferation were observed

When osteoblast proliferation on pristine implant surfaces was compared, proliferation was greatest on the smooth surface 7873 at all time points, followed by 7676 (smooth), then 7874 (nanorough) and was always lowest on 6905 (moderately rough) (Figure 34). The same trend was observed at all time points, however differences were only significant at days 7 and 14 Table 26.
Figure 34 HOB proliferation at different time points on different pristine implant surfaces. Highest proliferation rates were observed on surface 7873 (smooth) and lowest on 6905 (moderately rough) (n=6). Significant differences in proliferation are outlined in the table below.

<table>
<thead>
<tr>
<th>Day</th>
<th>Surface</th>
<th>Surface</th>
<th>p-value</th>
<th>Day</th>
<th>Surface</th>
<th>Surface</th>
<th>p-value</th>
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<tbody>
<tr>
<td>7</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<td>7874</td>
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</tr>
<tr>
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</tr>
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<td></td>
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<tr>
<td></td>
<td>7874</td>
<td>0.02</td>
<td></td>
<td>7874</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 26 Time points and surfaces at which significant differences in HOB cell proliferation were observed on pristine surfaces. Post Hoc Scheffe Test for multiple comparisons.
4.4.1.3 Osteoblast differentiation by Alkaline phosphatase assay

ALP activity was lowest after decontamination using BAG. This trend was observed on all surfaces at all time points and was shown to be significant at day 14 on surface 6905 after BAG compared to the same surface decontaminated using a scaler (p=0.006). Similarly, 7874 surfaces decontaminated using BAG at day 14 showed significantly lower ALP levels compared to using glycine (p<0.049). The only instance where ALP level was not the lowest after BAG decontamination was at day 28 on surface 6905. ALP levels were also significantly lower on control 6905 surfaces compared to those decontaminated with scalers at day 14 (p=0.012). However this was not repeated at any other time point or surface (Figure 35).
Figure 35 Alkaline phosphatase activity of alveolar osteoblasts cultured on different implant surfaces after being subjected to different decontamination methods.

Data is plotted as means ± standard deviation (n=6).
ALP activity was lowest after decontamination using BAG.
4.4.2 Surface Alterations caused by the Different Treatment Procedures

The morphologic characteristics after treatment procedures were investigated by means of SEM. After mechanical debridement, scratch marks were evident from the blade, which removed some of the original surface coating resulting in exposure of the titanium base underneath (Figure 36). Surfaces decontaminated using BAG blasting resulted in a surface that appeared homogenously rough regardless of original surface topography (Figure 37), while surfaces decontaminated with glycine powder blasting were covered with remnants of the rhomboidal – cylindrical powder particles, but did not seem to visibly alter the underlying titanium surfaces (Figure 38). The SEM images show both air powder abrasion systems to be more efficient in eliminating the attached biofilm compared to mechanical debridement, after which bacterial aggregates could still be observed.
Figure 36 SEM micrographs of surfaces treated by mechanical debridement (left x3000 magnification, right x6000 magnification). Scratch marks from mechanical debridement and remaining bacterial aggregates can be seen (Top to bottom: 6905, 7874, 7873, 7676)
Figure 37 SEM micrographs of surfaces treated by air-powder blasting using BAG (left x3000 magnification, right x6000 magnification). Surfaces appear homogenously rough regardless of original surface topography (Top to bottom: 6905, 7874, 7873, 7676)
Figure 38 SEM micrographs of surfaces treated by air-powder blasting using glycine (left x3000 magnification, right x6000 magnification) (Top to bottom: 6905, 7874, 7873, 7676)
Note the presence of rhomboidal – cylindrical glycine powder deposits.
The surface composition of the four discs after air-powder blasting was determined using EDS. The spectrum for discs decontaminated with BAG revealed the presence of calcium, phosphate and silicon, which are constituents of BAG indicating the inclusion of the particles within the titanium surface. Oxygen levels were comparable, but titanium levels were less than pristine surfaces (Figure 39).

![Elemental spectrum obtained by the EDS. Analysis of different titanium surfaces after decontamination using BAG blasting – Spectrum shows peaks for titanium and oxygen typical of these surfaces as well as peaks for calcium, phosphate and silicon which are constituents of BAG.](image)

<table>
<thead>
<tr>
<th>Element</th>
<th>Wt %</th>
<th>At %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti</td>
<td>27.13</td>
<td>11.26</td>
</tr>
<tr>
<td>O</td>
<td>43.92</td>
<td>54.59</td>
</tr>
<tr>
<td>Na</td>
<td>7.6</td>
<td>6.57</td>
</tr>
<tr>
<td>Si</td>
<td>5.3</td>
<td>3.75</td>
</tr>
<tr>
<td>P</td>
<td>0.66</td>
<td>0.42</td>
</tr>
<tr>
<td>Ca</td>
<td>1.81</td>
<td>0.9</td>
</tr>
<tr>
<td>C</td>
<td>13.59</td>
<td>22.50</td>
</tr>
</tbody>
</table>
After glycine (NH₂CH₂COOH) blasting, EDS analysis showed that oxygen levels were comparable, but titanium levels were lower compared to the pristine surfaces. Silicon and carbon were also found. Contrary to expectations, nitrogen and hydrogen were not detected. One explanation can be that Hydrogen gives off such a low energy x-ray and has no energy levels that it cannot be picked up by most EDS detectors used. The Nitrogen's K alpha energy is at 0.392 meaning that it could be a small peak overlapped and hidden in the area between the carbon (K alpha 0.277) and oxygen peak, possibly under the Titanium L alpha peak (L alpha 0.452) (Figure 40).

<table>
<thead>
<tr>
<th>Element</th>
<th>Wt %</th>
<th>At %</th>
</tr>
</thead>
<tbody>
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<td>O</td>
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<td>Si</td>
<td>08.87</td>
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<tr>
<td>Ti</td>
<td>04.69</td>
<td>01.48</td>
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</table>
Figure 40 Elemental spectrum obtained by the EDS analysis of different titanium surfaces after decontamination using glycine air blasting (from top to bottom 6905, 7874, 7873, 7676). Oxygen levels were comparable to the corresponding pristine surface, but titanium levels were lower. In addition, Silicon and Carbon were detected. The elemental constituents of glycine were not detected, however this could be due to the limitations of the EDS detector.

<table>
<thead>
<tr>
<th>Element</th>
<th>Wt %</th>
<th>At %</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>O</td>
<td>33.25</td>
<td>36.45</td>
</tr>
<tr>
<td>Si</td>
<td>05.16</td>
<td>03.22</td>
</tr>
<tr>
<td>Ti</td>
<td>27.05</td>
<td>09.90</td>
</tr>
</tbody>
</table>
4.5 Discussion

The removal of the bacterial biofilm and the cell components that might adversely influence host cell growth on implant surfaces seems to be essential for the treatment of peri-implantitis. Amongst various methods, air-powder systems have been shown to be a feasible alternative to conventional mechanical debridement for the elimination of biofilms from dental implants.

A powder of potential interest is glycine, which is a soft powder with documented safety to be used subgingivally. Research has shown that this powder induces the least gingival erosion compared to conventional sodium bicarbonate or hand instrumentation (Petersilka et al., 2008). In addition, it has been demonstrated that glycine was effective in removing biofilms from dental implants without any adverse surface effects. (Schmage et al., 2012, Schwarz et al., 2009a).

Another potentially interesting powder is BAG, which has been reported to be a successful osteoconductive and osteoinductive material (Froum et al., 1998). Furthermore, its use as a powder for the treatment of peri-implantitis has not been previously reported.

The primary purpose of the present in vitro study was to assess the influence of different air abrasive powders in re-establishing biocompatibility of S. mitis - contaminated implant surfaces. The results of the present in vitro study indicate that reestablishment of biocompatibility of implant surfaces was possible using any of the three methods tested, which were mechanical debridement, air-blasting using BAG and air-blasting using glycine.

No residual cytotoxicity could be detected and treated surfaces showed comparable results to sterile non-contaminated surfaces. Cell growth and proliferation was also observed on all surfaces regardless of the decontamination method. However, on smooth surfaces, cell proliferation was lower after air-blasting procedures compared to their control or mechanically debrided counterparts. One explanation could be the response of osteoblast cells to certain degrees of roughness, where it has been shown that osteoblast cell proliferation was greater on smooth surfaces (Martin et al., 1995, Isa et al., 2006). The air-
blasting procedures used could have resulted in slight roughening (however this was not measured) of these surfaces resulting in a reduced cell proliferation rate.

These present results corroborate, to a certain extent, the findings from an *in vitro* study by Schwarz et al. (Schwarz et al., 2009a) in which cell viability was established on SLA titanium surfaces treated with glycine powders. In their study however, mitochondrial activity of the cells was significantly lower compared to their untreated control titanium discs. This was not the case in the present study. This difference may be attributed to differences in cell type, assay or time point. In the study by Schwarz et al. (Schwarz et al., 2009a), human osteosarcoma SaOS-2 cells were used and transformed cell lines have different characteristics than primary cells. In the current study, primary alveolar HOB cells were chosen for the cell culture experiments due to their similarity to the types of cells that the implant surfaces would encounter during osseointegration. Furthermore, Schwarz et al. (Schwarz et al., 2009a) only assessed differences at day 7 and used a different ATP-based luminescent cell viability assay to measure mitochondrial activity. Another reason that they may have found lower cell viability could have been because surfaces were autoclaved after being treated with air blasting and prior to the cell culture experiments. Autoclaving has been shown to increase the carbon content of titanium surfaces, which in turn would affect cell viability (Park et al., 2010). They also found that powders that were harder and were able to produce a slight degree of surface ablation resulted in better cell viability. Again this was not the case in the present study, where using the harder BAG particles did not correlate with better proliferation. To the best of our knowledge, the study by Schwarz et al. (Schwarz et al., 2009a) was the only other study that has assessed biocompatibility after blasting using glycine.

We had postulated that the residual or embedded BAG particles would be released into the fluid with time due to their absorbable properties and that the ions initially released such as calcium and phosphate would play a direct role in improving the surface bioactivity and enhancing cell differentiation. Indeed, remnants of the blasting material were detected by EDS, which revealed the presence of calcium, phosphate and silica. However, results obtained from the ALP assay, showed that this did not result in increased cell differentiation. In fact, ALP activity was always lowest after decontamination using BAG blasting. This was significant on the rough surfaces, 7874 and 6905 treated with BAG-
blasting, which showed lower cell differentiation at day 14 compared to the same surfaces treated with glycine-blasting or a mechanical cleaning with a scaler respectively (see Figure 35).

Air polishing in this study was shown to be more effective in removing the attached biofilm compared to mechanical debridement. The efficiency of both these powders in this respect was demonstrated using SEM, although for the glycine powder there was a possibility that the powder remnants obscured underlying bacterial aggregates (Figure 38). This is consistent with other studies that reported that air polishing delivered the cleanest surfaces (Schmage et al., 2012) and removed 100% of the biofilm from SLA titanium surfaces (Schwarz et al., 2009a). This cleaning efficiency could be more pronounced in vivo where access for mechanical debridement could be more problematic.

The amount of bacterial or powder remnants that remained on the titanium surfaces did not seem to affect the biocompatibility of the surfaces. Despite the presence of observed bacterial colonies after treatment with mechanical debridement for example, growth of vital HOB cells was still observed on all surfaces treated using this method. It is unknown to what extent bacterial and non-bacterial residues have to be removed from an implant surface in order to obtain a predictable, stable clinical result after treatment (Mombelli, 2002)

Although, it was not one of the aims of the study to measure surface alterations caused by the different decontamination instruments, SEM observations showed surface alterations after BAG blasting and after scaling. It is debatable whether surface alterations would affect re-osseointegration. McCollum et al. (McCollum et al., 1992) states that de novo plaque accumulation was similar regardless of surface texture of abutments after being treated with different instruments. However, some authors recommend using instruments that cause least surface alterations (Schmage et al., 2012, Schwarz et al., 2009a), whereas others recommend burnishing and polishing exposed implant threads (implantoplasty) to aid in the treatment of peri-implantitis (Lang et al., 2000).

Due to BAG’s highly abrasive nature, an obvious dulling of the surfaces was observed. In addition sparks were produced during the collision of the glass particles with the titanium surfaces. Thus, BAG seems to be too abrasive to be recommended for use in deep peri-
implant defects and may be associated with an increased risk of soft tissue trauma. Other studies have stated that BAG can remove enamel at half the volume of a slow speed tungsten carbide bur (Banerjee et al., 2008). A recommendation could be to reduce the pressure/flow settings when using BAG but this was not tested in the present study and more research is needed to support the safety of BAG powder in air polishing in general and subgingival air polishing in particular.
CHAPTER 5  GENERAL DISCUSSION
Implant dentistry has progressed significantly in terms of surgical techniques and surfaces that accelerate osseointegration, however when peri-implantitis is diagnosed, there are no predictable treatments to resolve the problem (Donos, 2012). In 2008, The 6th EWOP (Lindhe et al., 2008) came to the conclusions that peri-implantitis lesions were caused by bacteria and that it was difficult to treat, warranting research in the microbiota making up the peri-implantitis community and the methods used for implant surface decontamination. In addition, there was conflicting evidence showing that different surfaces of implants may be more susceptible to peri-implantitis (Heitz-Mayfield, 2008).

5.1 THE MICROBIOTA ASSOCIATED WITH PERI-IMPLANTITIS

A complex and heterogeneous subgingival microbiota was generally found around dental implants that have been in function for more than one year.

Peri-implantitis was accompanied by an increased bacterial load, with the phylum Firmicutes, which are mainly Gram-positive organisms, making up the greatest proportion of this load. On the species level, F. alocis, S. constellatus, P. micra and A. meyeri were consistently found in greater proportion in these sites suggesting that these Gram-positive anaerobes and facultative anaerobes may play an important role in the pathogenesis of the disease. This is a very different picture to that described by previous studies which targeted the presence of specific bacteria. In those studies significantly higher proportion of Gram-negative anaerobic bacteria were reported in peri-implantitis (Alcoforado et al., 1991, Leonhardt et al., 1999, Mombelli et al., 1987, Listgarten and Lai, 1999, Rams et al., 1984, Charalampakis et al., 2012). Our results, however, are supported by recently published studies that have used an open-ended approach to describe the peri-implant bacterial community (da Silva et al., 2013, Koyanagi et al., 2013, Tamura et al., 2013b).

This highlights the importance of using such an approach when studying bacterial communities especially if treatments involving antimicrobials are proposed. The current results suggest that a broad spectrum antibiotic, which covers both aerobes and anaerobes should be prescribed in the treatment of peri-implantitis. This supports the study by Rams et al. (Rams et al., 2014) who found that peri-implantitis patients frequently yielded submucosal bacterial pathogens resistant to individual therapeutic concentrations of
clindamycin, amoxicillin, doxycycline, or metronidazole, but only rarely to both amoxicillin and metronidazole.

Species that were isolated in significantly greater proportions in the peri-implantitis samples are considered in the following section.

5.1.1 Filifactor alocis

*F. alocis* is commonly being reported in recent studies as an important species that is strongly associated with oral diseases including periodontitis (Abusleme et al., 2013, Griffen et al., 2012) and peri-implantitis (da Silva et al., 2013, Tamura et al., 2013b). In the present study, it’s prevalence in peri-implantitis ranked higher than traditionally established pathogens such as *P. gingivalis*. It was consistently isolated in nearly every sample with peri-implantitis and in none of the healthy samples. *F. alocis* are Gram-positive, asaccharolytic, obligate anaerobic rods. They were initially identified in 1985 as *Fusobacterium alocis* and reclassified in 1999 into *Filifactor* according to their observed phylogenetic relationship based on the 16S rRNA gene (Jalava and Eerola, 1999). A recent study evaluating the virulence properties of *F. alocis* and it’s ability to interact with *P. gingivalis* have reported that *F. alocis* was more resistant to oxidative stress and that its growth was stimulated under oxidative stress when compared to *P. gingivalis*. Sialidase activity, which is important for survival, persistence and pathogenesis of periodontal pathogens, was also present in all the *F. alocis* strains tested, but was 40% less than *P. gingivalis*. Last but not least, cocultures of *P. gingivalis* and *F. alocis* revealed enhanced biofilm-forming capacity and enhanced invasion and adhesion of epithelial cells (Aruni et al., 2011). The fastidious nature of *F. alocis* has been implicated as the reason for its lack of being detected by culture studies and hence being overlooked as an important periodontal pathogen, however this was not the case in our in our study, where it was cultured without difficulty. Schlafer et al. (Schlafer et al., 2010) have also detected the species in 77% of subjects with chronic periodontitis and 78% of those with generalized aggressive periodontitis, reflecting on the importance of *F. alocis* in periodontal disease. Its prevalence was on a par with the organisms considered to be key players in periodontal disease, but was the lowest compared to the same pathogens in healthy individuals (Schlafer et al., 2010). This is similar to our current findings in peri-implantitis patients and is confirmed by other recent reports that describe the peri-implantitis community (da Silva et al., 2013,
Tamura et al., 2013b). Moreover, the close colocalization of *F. alocis* with periodontal pathogens that was observed in an *in vivo* biofilm model may indicate that these species are important in coaggregation events that take place during the establishment and maturation of biofilms (Schlafer et al., 2010).

### 5.1.2 *Parvimonos micra*

The significantly increased proportion of *P. micra* in peri-implantitis in the present study as well as all the other studies mentioned in table 17 seems to implicate them strongly in peri-implantitis (Tamura et al., 2013b, da Silva et al., 2013, Koyanagi et al., 2013, Kumar et al., 2012). In addition, they were detected by Rams et al. (Rams et al., 2014) in 111 out of 120 patients with peri-implantitis and by Alcoforado et al. in a failing implant with inflammation (Alcoforado et al., 1991) when the species was formerly known as *Peptostreptococcus micros*. They are obligate, asaccharolytic, anaerobic, Gram-positive, and non-spore-forming bacterial species that are part of the ‘orange complex’ of species (Socransky et al., 1998) and have been described in oral health as well as in relation to a variety of oral diseases such as periodontitis (Rams et al., 1992), endodontic infections (Siqueira et al., 2007) and dentoalveolar infections.

### 5.1.3 *Streptococcus constellatus*

*S. constellatus* is also a member of the ‘orange complex’ of species (Socransky et al., 1998). They are Gram-positive, non-spore forming, non-motile cocci, which are part of the milleri group. They have been reported to be more common in subgingival plaque than the other two members of the milleri group (*S. intermedius* and *S. anginosus*), which were more common in supragingival plaque (Whiley et al., 1990). *S. constellatus* were the most numerically elevated streptococci in refractory periodontitis in patients with poor response to scaling and root planing (SRP) (Colombo et al., 1998) and have been commonly associated with abscesses (Claridge et al., 2001) and dental infections (Whiley et al., 1990). Regarding dental implants, Tamura et al. support the findings of this study regarding the importance of *S. constellatus* in peri-implantitis, which they found in high proportions in their failing sites, but were not detected in any of the 15 healthy implant sites. They also noted that although streptococci were the predominant genus in both peri-implantitis and health, the species in both presentations were not always the same. For instance, *S. sobrinus*
were found only in peri-implantitis in their study and our findings concur with their results as we only found \textit{S. sobrinus} in the peri-implantitis samples.

5.1.4 \textit{Actinomyces meyeri}

In contrast to our findings, Tamura et al. (Tamura et al., 2013b) found that \textit{A. meyeri} predominated in healthy sites. There was no mention of \textit{A. meyeri} in the other studies. In an \textit{in vitro} biofilm study, \textit{A. meyeri} was the most prominent colonizer on various titanium surfaces compared to \textit{A. naeslundii, A. odontolyticus} and \textit{A. israelii} (Sarkonen, 2007). The same authors found that \textit{A. odontolyticus} was the most prominent species isolated from 33 failing implants (Sarkonen et al., 2005). \textit{A. odontolyticus} and \textit{A. meyeri} are closely related phylogenetically and biochemical testing may have been necessary for accurate phenotypic differentiation of the two. There is a possibility that the species identified in this study may be \textit{A. odontolyticus} and that would coincide with the observations of Sarkonen et al. (Sarkonen et al., 2005). On the other hand, the presence of a considerably high proportion of \textit{A. meyeri} would reinforce the fact that they are important initial colonizers in the process of biofilm formation on dental surfaces. However what was surprising was the total absence of \textit{A. meyeri} in healthy sites in this study.

Traditionally, \textit{A. meyeri} has been described as a facultative anaerobe isolated from gingival crevices of periodontally healthy individuals. However, it has also been isolated in samples from active destructive periodontitis (Moore and Moore, 1994), brain abscesses, liver abscesses (Felekouras et al. 2004) and chronic pancreatitis (Harsch et al. 2001).

It is important to remember that the mere isolation of bacteria from sites with disease is not enough to understand the complex pathogenesis of peri-implantitis and it was not the aim of this study to try to pinpoint specific species, but rather to describe and compare the bacterial community surrounding failing and healthy dental implants.
5.2 PROGRESSION OF PERI-IMPLANTITIS ON DIFFERENT SURFACES

In the current study, peri-implantitis was observed more around implants with a moderately roughened surface compared to those with a machined surface, however there was no intention to investigate the prevalence of peri-implantitis according to the different surface textures or manufacturer, as this would have required a much larger and preferably randomized study. In the literature, there is inconsistent evidence to suggest that implant surface may be a risk indicator for peri-implantitis (Heitz-Mayfield, 2008). A retrospective study which compared 2,182 smooth-surface implants with 2,425 rough-surface implants found no difference in the survival rates of these implants (Balshe et al., 2009), while a Cochrane systematic review that compared the occurrence of peri-implantitis between turned and roughened surfaces showed that more implants with rough surfaces were affected by peri-implantitis over a 3 year period and that turned machined implants had 20% less risk of being affected. These findings were based on 3 randomized clinical trials, with relatively few participants, often with a risk of bias and the authors concluded that there was no evidence to show that any particular type of implant has superior long-term success (Esposito et al., 2007).

5.3 BIOFILM FORMATION ON IMPLANT SURFACES

The first generation of implants were mainly concerned with improving mechanical properties, while the current generation of implants have focused more on surface modifications both topographically and chemically. It seems that the development of the future generation of implants will be aimed at surface modifications using pharmacological components and molecules to direct and enhance cellular interactions (Palmquist et al., 2010b).

Of particular importance is the possibility of surfaces in minimizing biofilm formation. In the present study, no differences observed in biofilm formation after 96 hours between any of the moderately rough surfaces (with or without fluoride) or the smooth surfaces used. It appears that once initial colonization has been established, the surface has little effect thereafter. Again the findings of the current study are quite different from earlier studies in
which rougher implant surfaces were commonly associated with increased biofilm formation (Bollen et al., 1996, Quirynen et al., 1993, Quirynen and Listgarten, 1990), however corroborate to a large extent the results of recent studies on biofilm formation (Schmidlin et al., 2013, Lin et al., 2013, Frojd et al., 2011b, Frojd et al., 2011a, Al-Ahmad et al., 2010) and clinical studies on progression of peri-implantitis on moderately rough implant surfaces (Wennstrom et al., 2004, Astrand et al., 2004b), in which no differences could be found regardless of the surface (moderately rough or smooth) used.

5.3.1 In Vitro Biofilm Model

It could be argued that this study used a single-species biofilm that is associated with healthy implant sites. However, as previously mentioned, streptococci are important early colonizers (Marsh 1995, Li 2004) and make up a major component of a biofilm (Al-Ahmad 2010). It is their adhesion, which facilitates the colonization of subsequent organisms that would otherwise be incapable of binding to host surfaces and could ultimately lead the formation of a pathogenic biofilm. A single species was used because it was found to yield more standardized results in term of bacterial counts than when a multispecies biofilm was attempted (Appendix 5). Other studies have shown very different bacterial adhesion when a 2 species (S. sanguinis + A. naeslundii) and a 3 species (S. sanguinis + A. naeslundii + L. salivarius) biofilm were grown in vitro (Frojd et al., 2011a). Conversely biofilm formation and virulence expression by S. mutans were altered when grown in a dual-species model (Wen et al., 2010), whereas V. dispar seemed to have profited from the lactic acid produced by the streptococci and continued to grow increasingly up to the 16.5 hours of the experiment, whereas others only showed minimal increase or remained stagnant (Schmidlin et al., 2013). These studies emphasize the fact that in addition to the characteristics of the surface, the model bacteria used will influence the biofilm formation on titanium surfaces in vitro (Frojd et al., 2011a). In this study it was essential to keep confounding factors to a minimum and thus it was agreed that a single species would be most suitable.

The in vitro model system was based on the previously validated Zurich biofilm model described by Guggenheim et al. (Guggenheim et al., 2001). Various models of in vitro oral biofilm models have been described and applied to problems of clinical relevance. These models can be classified according to the type of delivery of the growth medium into two main types, dynamic and static. Dynamic models or fluid displacement systems consist of
either flow cells (Larsen and Fiehn, 1995, Christersson et al., 1987) or of chemostats (Bradshaw et al., 1996) modified to allow insertion and removable of colonizable surfaces and utilize fluid flow or scraper blades to generate continuous detachment forces of the biofilm. Examples include the artificial mouth (Sissons et al., 1991) and the constant depth film fermenter (CDFF) flow cells, which has been widely used in oral biofilm development and research (Pratten, 2007, Peters and Wimpenny, 1988).

While these devices have contributed to our understanding of biofilm adhesion and formation, their use has certain drawbacks. They are complex instruments that involve different types and sizes of vessels, tubes and valves. This complex structure leads to difficulties in ensuring sterile conditions are maintained throughout the duration of the experiment. In addition, they require a thorough knowledge of the device assembly, hence they are technically demanding and difficult to adjust and control. Furthermore, most of the time, limited number of samples can be used and sometimes these samples need specific dimensions. Moreover, the pulsed particles are cleared from the culture by the action of the flow rate and volume. As a result, chemostats with large volumes and relatively slow flow rate could mean long residence times, making them inappropriate for studying agents with short exposures. For example, if it were decided to test the effect of different antimicrobial rinses applied for 30 seconds on the biofilms grown on the titanium implants, this would have not been possible. In addition, artificial models that use a working volume of more than a few millimetres would not allow the use of media made up of natural substrates such as human saliva (Guggenheim et al., 2004).

Static models on the other hand such as the microtitre plate model used in this study or the minimum biofilm eradication concentration assay are simple to prepare, maintain and analyze and do not require specialized laboratory equipment. They allow convenient changes of experimental parameters (species, medium, additives), which can be applied and removed from the system virtually instantaneously if necessary. Drawbacks of these models include that the biofilms cultivated are subjected only intermittently to detachment forces in the form of dip-washing or gentle swirling. In the present study, these forces were enough to dislodge the loose bacteria surrounding the biofilm, but not the firmly attached cells from the disc surfaces. In addition, such systems are closed without in or outflow during the experiment (Heersink and Goeres, 2003). As a consequence, the environment in the well of
static biofilm model will change during the experiment (e.g. nutrients become depleted, signalling molecules accumulate, etc.) unless the fluid is regularly replaced and replenished, as was done in the current study.

An important parameter of the *in vitro* model is the medium used. In the current study we used a medium containing serum. The aim was to reflect the state of the titanium surface when implanted *in vivo* since one of the earliest events that occurs during the placement of dental implants is the coating of the titanium surface with serum proteins. This happens as the implant contacts blood at the implant site and is coated with the abundant cell attachment proteins. Consequently, using a medium containing serum was deemed to reflect the clinical situation more accurately than using a serum free medium.

### 5.3.2 Titanium Surfaces (*Appendix 6*)

One mechanism that is used to increase bone to implant contact and to promote rapid osseointegration is to increase implant surface roughness (Steveling et al., 2001). The moderately roughened titanium surfaces used in this study have been well documented in the clinical scientific literature and have an excellent history of successful osseointegration (Rasmusson et al., 2005). The TiOblast™ surface (disc reference number 6905) was introduced in 1990 and is a titanium surface that has been grit blasted with titanium dioxide particles to achieve a moderately rough surface (Sa ≈ 1.1 ± 0.2 µm). It has shown excellent outcomes when compared to machined titanium in terms of *in vitro* cell biocompatibility (Mustafa et al., 1998), *in vivo* histomorphometrical comparisons and human histological (Ivanoff et al., 2001) and clinical studies (Gotfredsen et al., 1992). In a clinical study, only minor marginal bone level alterations of 0.3 mm were recorded during a 5-year follow up (Palmer et al., 2000). The OsseoSpeed™ surface (disc reference number 7874) is a development of the TiOblast™ surface and was introduced in 2004. It is characterized by the incorporation of small amounts of fluoride ions in the oxide layer and a slight topographic modification resulting in the appearance of nanometer-sized peaks and a slight increase in the micrometer scale of surface roughness (Sa ≈ 1.5 ± 0.07 µm). *In vitro* and animal studies (Berglundh et al., 2007a, Ellingsen et al., 2004, Guo et al., 2007, Isa et al., 2006) have indicated increased bone-to-implant bonding at shorter healing times compared to TiOblast™ and there was no significant dip in Implant Stability Quotient, often seen with implants 2 – 6 weeks post-insertion. If we take into account the increased surface area on
these surfaces and the tendency of lesser biofilm formation patterns, it may be postulated that fluoride might have contributed to this.

The machined titanium surface 7676 (Sa ≈ 0.2 ± 0.05 µm) is the base surface, which is either polished to be used as an abutment surface that is smooth (as seen in the SEM) or is blasted to produce the implant surfaces TiOblast/ Osseospeed.

Finally, the smooth test surface 7873 (Sa ≈ 0.16 ± 0.15 µm), was a titanium surface made of Grade V CP Ti. This surface is used for different products, but not for the implants.

To exclude the possibility that any differences seen in biofilm formation could be attributed to surface wettability, all surfaces used in this study exhibited a comparable contact angle of 90 degrees and were considered slightly hydrophobic.

5.3.3 Biofilm Analysis

In this study, biofilm analysis was achieved using 3 different methods; traditional bacterial culture count, SEM which provides high resolution imaging and good depth of field but requires dehydration and fixation of the biofilm and CLSM which enabled a non-invasive evaluation of the hydrated biofilms and allowed 3D imaging of fluorescent data, by taking a series of XY images and superimposing them on one another in the Z-axis. The Leica SP2 used is an inverted laser scanning confocal microscope and because the scope is inverted, much thicker specimens can be imaged, compared to an upright scope, making it ideal for live imaging.

Using CLSM, titanium reflection proved to be difficult to overcome and could have produced a procedural error, however every effort was done to minimize reflection by using a negative control to omit background reflection before the start of each imaging session and adjusting the start point of biofilm analysis (approximately 2 µm away from the disc surface). Also all discs for the same experiment were imaged in the same session directly after staining to avoid any adverse storage effects on the biofilm.

An alternative to using CLSM imaging titanium could be the use of the 2-photon microscope. The concept of the 2-photon is that a laser with double the wavelength required for fluorophore excitation is used. i.e. The energy of 2 photons are necessary for
excitation. For example, if the intention were to excite a fluorophore of a wavelength of 400 nm, a laser with 800 nm would be necessary for excitation. The reflected light will be 800 nm, however the fluorescence from the excited fluorophore would be much less than 800 nm, consequently, there is much wider separation between the wavelength of the reflected and the fluorescence, making images much clearer when there are reflection problems as in titanium.

Regarding image analysis, most colour image analysis methods use the original confocal image data file to perform a monochromatic segmentation of the image. The software used in our study enabled in situ colour segmentation without prior transformation of the micrographs into monograph channels. This colour segmentation relies on the colour additive theory, which classifies each pixel of the image into predefined colour classes. The colour to be segmented is identified by green, red, and blue codes which are then subtracted from each pixel in the image enabling differentiation between live and dead subpopulations of the biofilm.

5.4 The effect of various decontamination methods on re-establishing biocompatibility of contaminated implant surfaces

The most common method to debride implant surfaces and remove the plaque biofilm is to use manual or ultrasonic scalers. However, accessibility problems and inherent difficulties for implant debridement imposed by implant threads and surface roughness may hinder effective cleaning. APDs may overcome some of these difficulties if direct access to the surface cannot be achieved (Meyle, 2012).

In the present study, three decontamination methods were evaluated in their ability to re-establish biocompatibility of the implant surface. These were mechanical debridement and air polishing using a glycine powder or BAG. The first powder was chosen in order to minimize surface alterations that may occur during air polishing, since glycine is characterized by a small particle size with low abrasiveness, while BAG was chosen to deliver bioactive molecules on the implant surface.

To assess biocompatibility in vitro, two types of cells are generally used, primary cells and cell lines. Primary cells are advantageous for studies of bone cell metabolism and
differentiation because they retain a normal phenotype since no dedifferentiation has taken place (Aubin et al., 1995, Di Silvio and Gurav, 2001). In the current study, it was decided to use primary cells characteristic of the implant site (i.e. alveolar HOB cells).

Results of the current study indicated that regardless of the decontamination method used, HOB cells cultured on the implant surfaces showed that all discs, allowed cell attachment, cell proliferation, and osteoblastic differentiation after decontamination and their biocompatibility was comparable to pristine surfaces. However, air-polishing using BAG or glycine achieved greater surface cleanliness compared to mechanical debridement using a curette.
5.5 Limitations of this Research

5.5.1 Study 1

1) This study was culture-based, and thus did not detect phyla (e.g., TM7, Synergistetes and Spirochaetes) and species that may either be unculturable or difficult to detect with culture based methods. It is important in order to obtain a comprehensive representation of the diversity around implants to combine a similar culture study with one that is not non-culture dependent. Da Silva et al. stated that the not-yet-cultivated bacterial species accounted for 32% - 40% of their results, but there were no observed differences in the mean proportion of these bacteria in peri-implantitis and health (da Silva et al., 2013). Recently, next generation pyrosequencing technology has been introduced that has enabled researchers to examine thousands of sequences from several samples simultaneously, providing a more comprehensive coverage than was previously possible. The only study that has done this in peri-implantitis is by Kumar et al. (Kumar et al., 2012) and has only discussed phylum and genus level results. They have reported a very different microflora to the present study and to others employing an open-ended approach (Tamura et al., 2013b, Koyanagi et al., 2013, da Silva et al., 2013) (Table 17). Further investigations are necessary to confirm their findings and to distinguish taxa at the species-level, as different species within the same phylum and/or genus may be health-associated or disease-associated.

2) Bacterial identification was carried out using 16S rRNA gene sequencing. A limitation of this method is its inability to discriminate between closely related species and when identity match is 99% or more. In the present study, when multiple hits above 98.5% were identified with HOMD, all possibilities for species were reported. In addition, this limitation was explored further in the following two situations.

i) Differentiation of S. constellatus and intermedius using the sialidase test

In 1990, Beighton and Whiley (Whiley et al., 1990) described a simple method, using the sialidase (neuraminidase) activity of bacterial strains and based on the use of the fluorescent substrate (4-methylumbelliferyl a-D-N-acetylneuraminic acid). Using this test, they showed that strains of S. intermedius could be easily distinguished from S. constellatus and S. anginosus, by the strongly positive results for the production of sialidase by S.
intermedius. In the present study, 16S sequencing using the forward primer was confirmed to be a reliable method to differentiate between these two closely related species.

**ii) Met-G PCR Sequencing analysis for Actinomyces**

Identification of *Actinomyces* species is problematic. Furthermore, strains of genospecies 1 and 2 exhibit > 99% 16S rRNA gene sequence similarity (Henssge et al., 2009). Henssge et al. (Henssge et al., 2009) showed that using the partial sequences of atpA and metG gave the best separation of the two species, where *A. naeslundii* genospecies 1 and 2 formed two distinct clusters. Thus, *A. naeslundii* genospecies 2 became *A. oris sp. nov* and *A. naeslundii* genospecies 1 remained as *A. naeslundii sensu strictu*. The present study confirmed that 16S rRNA gene sequencing was not the most reliable method for the identification the *A. naeslundii* genospecies.

3) Regarding sampling technique; the advantage of using curettes as opposed to paper points is that the latter may sample only a small fraction of the bacterial biofilm present in the peri-implant pocket, possibly underestimating the presence of certain species (Gerber et al., 2006). For instance, rough surfaces harboured 25 times more bacteria than smooth surface when curettes were used to obtain plaque samples, whereas when paper points were used, minor differences were observed (Quirynen et al., 1993). The paper point sampling technique may also recover less cells of species adherent to tooth surfaces such as *Actinomyces* and increase the proportion of organisms adherent or loosely adherent to the soft tissues (Quirynen et al., 2006). On the other hand, using paper points is a more standardized procedure and combining both sampling methods would be optimum.

Another issue to bear in mind regarding sampling from around implants is that it could be technically challenging, especially if using paper points, due to implant-crown contours and implant surface configurations. Ideally it would be best if the supra structure was removed first, however this is not always practically feasible.

**5.5.2 Study 2**

Intraoral biofilm formation is a complex process which involves hundreds of different species and cannot be reproduced *in vitro* and care should therefore be exercised in extrapolating the results of *in vitro* experiments to the *in vivo* situation. *In vitro* studies are
more easily standardized, more reproducible and provide results that are easier to interpret than in vivo studies. The latter have the advantages of providing more realistic conditions (complex plaque), however with many variations and confounding factors (salivary content, salivary forces, soft tissue movement, dietary habits, oral hygiene habits, etc.). Further clinical investigations are required to determine if surface characteristics affect the properties of the attached microbial biofilms.

5.5.3 STUDY 3

1) It was necessary to gamma radiate the discs to inactivate bacteria and provide a sterile surface in order to permit cell culture assays. This is not a clinical possibility for treating implants associated with peri-implantitis associated and indeed the cell response could have been different if gamma radiation was not performed. On the other hand, this inactivation of bacteria alone should not have been insufficient to re-establish a biocompatible surface since it is necessary to also remove cell components which would adversely affect cell growth on implant surfaces (Kreisler et al., 2005). It would have been interesting to use a contaminated surface that was only gamma radiated.

2) No water was used during operation, which does not reproduce the settings in clinical practice. On one hand, water has been considered to improve substance removal by acting as an acceleration medium for particles. On the other hand, it has been proposed that water may reduce the impact effect of abrasive particles (Petersilka, 2011). The intention was to compare BAG vs. glycine vs. mechanical scraping and limit the effect of any other confounding factors. Nevertheless, this still makes it different than the intended use in a clinical setting.

3) Finally, from a clinical point of view, an in vitro single species biofilm collected after 96 hours is non-mineralized and is very different from subgingival plaque and calculus that would be observed in vivo.
5.6 CONCLUSIONS

5.6.1 STUDY 1

- Peri-implantitis is a polymicrobial, complex and heterogeneous infection that is accompanied by an increased bacterial load.

- Various species, namely *F. alocis, P. micra, S. constellatus* and *A. meyeri* were predominantly isolated in peri-implantitis sites. These are not the so-called ‘periodontal pathogens’ that were traditionally associated with periodontal disease, however they were more dominant than the latter and seem to play an equally, if not more important role while *V. parvula/dispar* and *N. elongata* were more prevalent in health.

5.6.2 STUDY 2

- Within the limitations of the current study, our findings indicate that titanium surfaces that were different topographically or chemically did not have any differential effects on a single-species biofilm accumulation after 96 hours. It appears that once initial colonization has been established, the effect of the surface diminishes with maturation of the biofilm.

- Most current implant surfaces have some sort of chemical treatment after the initial micro roughening to enhance osseointegration. From a clinical point of view, this could indicate that surfaces currently available will not increase biofilm accumulation and the most important factor in preventing peri-implantitis seems to lie in the patient’s ability to maintain proper oral hygiene.

5.6.3 STUDY 3

- Although the present results cannot be extrapolated directly to the clinical setting, the present study indicates regardless of the decontamination method used biocompatibility of the surfaces was established and was comparable to their corresponding pristine surfaces.
• No added bioactive beneficial effects were observed after BAG-blasting. In addition, its abrasive nature may contraindicate its safe use in subgingival air polishing. On the other hand glycine was shown to achieve a biocompatible surface similar to the other decontamination methods, but its low abrasiveness and documented safety would support its use as an adjunct cleaning method after the removal of mineralized deposits and during the maintenance phase for peri-implantitis.
5.7 Future Considerations

5.7.1 Study 1

- Organisms detected around implants with peri-implantitis, especially *F. alocis* could be considered as disease-associated organisms and warrant further research.

- It has been suggested that butyrate plays an important role in local tissue destruction by Tamura et al. (Tamura et al., 2013b) who reported elevated levels of butyrate-producing *E. nodatum, Eubacterium saphenum* and *Eubacterium minutum* in peri-implantitis. Conversely, Kumar et al. (Kumar et al., 2012) detected *Butryvibrio fibrisolvens*, a butyrate producer in the human gastrointestinal tract in all peri-implant crevices. Interestingly, *F. alocis* which were predominantly increased in peri-implantitis in the current study are butyrate producers. How this could be related to the pathogenesis of peri-implantitis maybe a possible aspect that could be worth investigating.

- It is important in order obtain a comprehensive representation of the diversity around implants to combine a similar culture study with one that is not non-culture dependent.

5.7.2 Study 2

- Using an *in vivo* plaque sample or using patient’s saliva to form a multispecies *in vitro* biofilm, could reflect more realistic clinical conditions than the current study.

- A comparison of the biofilm characteristics during the initial stages of biofilm formation on the different surfaces could also be of interest.

5.7.3 Study 3

- To date only two clinical studies (Renvert et al., 2011, Sahm et al., 2011) are available for glycine air-powder polishing for peri-implantitis. Both have showed limited clinical improvements. However both studies were non-surgical and non-surgical treatment for peri-implantitis has been deemed ineffective in the Sixth European Workshop on Periodontology (Lindhe et al., 2008). Clinical studies are
therefore justified to evaluate the applicability and efficacy for glycine air-powder blasting for the treatment of peri-implantitis
DISCLAIMERS AND CONFLICT OF INTEREST

The authors declare that they have no conflict of interests. The titanium discs were kindly provided by Astra Tech (Dentsply Implants, Astra Tech, Mölndal, Sweden) and the Airflow® handy 2+ hand piece and the Airflow® subgingival PERIO powder were kindly provided EMS (Electro Medical Systems, UK).
REFERENCES


("Streptococcus milleri group") are of different clinical importance and are not equally associated with abscess. *Clin Infect Dis*, 32, 1511-5.


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**Appendices**

**Appendix 1 ADI Algorithms for Treatment of Peri-implant Disease**

Non-surgical Algorithm for Treatment of PID:

- **Non-surgical Treatment**
  - Remove risk factors

- **Prosthetic Design**:
  - Retained cement
  - Non-passive fit of restoration
  - Occlusal scheme
  - Uncleansable restoration

- **Patient factors**:
  - Smoking
  - Stress
  - Poor oral hygiene

- **Susceptibility**:
  - Uncontrolled diabetes

- **Antimicrobial mouthwash**
  - 0.2% Chlorhexidine for 3-4 weeks

- **Is there any CBL?**
  - Yes
  - Consider removing restorative component
  - Debride implant sulcus with a titanium or metal hand scaler
  - **Topical Antibiotics** into sulcus
  - Minocycline
  - Tetracycline

  - **Systemic Antibiotics**
    - 250mg Amoxicillin TDS for 10 days and 200mg Metronidazole TDS for 10 days.
    - (250mg Erythromycin QDS for 10 days for penicillin allergy)

- **Review in 6 weeks**
  - Resolution:
    - PPD <3mm
    - No BOP
    - No CBL
  - No
  - Yes

- **Non-surgical Algorithm**

- **Review in 6 weeks**
  - Resolution:
    - PPD <3mm
    - No BOP
    - No further CBL
  - Yes
  - No

- **Maintenance Algorithm**

- **Surgical Algorithm**

*Please refer to the ADI Guidelines on Peri-implant Monitoring and Maintenance for this algorithm.*
Surgical Algorithm for Treatment of PID:

1. **Surgical Treatment**
   - Is there sufficient residual bone? 
     - Yes
     - Remove risk factors
     - Antimicrobial mouthwash
     - Systemic antibiotics
     - Consider removing the restorative component
   - No
     - **Explantation**

2. **Prosthetic Design**
   - Retained cement
   - Non-passive fit of restoration
   - Occlusal scheme
   - Uncleansable restoration

3. **Patient factors**
   - Smoking
   - Stress
   - Poor oral hygiene

4. **Susceptibility**
   - Uncontrolled diabetes
   - 0.2% Chlorhexidine for 3-4 weeks

5. **Start 24 hours Prior to Surgery**
   - 250mg Amoxycillin TDS for 10 days and
   - 200mg Metronidazole TDS for 10 days
   (250mg Erythromycin QDS for 10 days for penicillin allergy)

6. **360 degrees surgical debridement and decontamination of implant surface**
   - 1st Choice: Titanium/Metal scalers
   - 250mg Tetracyline slurry
   - Consider: Laser
   - Hydrogen Peroxide
   - Chlorhexidine
   - Air-Abrasion
   - Citric Acid

7. **Regeneration of lost tissue**
   - 1st Choice: Bovine bone mineral and collagen membrane
   - Consider: No regeneration
   - Implantoplasty
   - Pocket reduction
   - Free gingival graft
   - Apically repositioned flap
   - Ethical/religious belief

8. **Review in 6 weeks**
   - Resolution: PPD < 3mm
     - No BOP
     - No
     - **Explantation**
   - Yes
     - **Maintenance Algorithm**

9. **PPD**
   - BOP / Exudate
   - Mobility
   - OH
   - Prosthesis stability / occlusion
   - Peri-implant tissue – Tone, texture, colour
   - PA radiograph

10. ***Please refer to the ADI Guidelines on Peri-implant Monitoring and Maintenance for this algorithm.*
APPENDIX 2 DETERMINATION OF SAMPLE SIZE

Preliminary statistical analyses were carried out after samples were obtained from 13 patients. Species that showed significant or near significant differences ($p \approx 0.05$ or less) are outlined in Table 27. After evaluating the results, it was decided that double the current sample size would be necessary to determine whether or not these observed differences would be maintained. It was concluded that the target number of patients would be a minimum of 20 and a maximum of 24. This would ensure that the study had sufficient clinical and scientific value and would be appropriate to the amount of time and resources available (Needleman et al., 2012).

<table>
<thead>
<tr>
<th>Species</th>
<th>Diagnosis</th>
<th>$p$-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy</td>
<td>Peri-implantitis</td>
</tr>
<tr>
<td>Filifactor alocis</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Neisseria elongata</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Fusobacterium nucleatum</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Terrahaemophilus aromaticivorans</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Parvimonas micra</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Streptococcus constellatus</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Veillonella parvula</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Prevotella intermedia</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Actinomyces meyeri</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 27 Number of samples in which each species was detected. The species in the table represent those where $p$ values $\leq 0.05$ (chi squared test)
APPENDIX 3 PATIENT DATA SHEET

Consent Form ☐ Sample Number ☐ Patient Number ☐

Date patient Referred .................................................................
C/O........................................................................................................
M.H........................................................................................................
D.H........................................................................................................
- Smoker Yes ☐ No ☐ Previous Pack Years........
  - No Antibiotics taken during the past month ☐
  - No Anti inflammatory taken during the past month ☐
  - No perio treatment during the past month ☐

**Implant**
Type Branemark ☐ Straumann ☐ Astra tech ☐
Location ............................................................................................
Length/ Diameter ..................................................................................
Surface characteristics ...........................................................................
Comments ............................................................................................

**Prothesis**
Type: Single/ multiple ☐ Cement/ screw ☐
Year dental implant treatment completed ...........................................
Date peri-implantitis first recorded ....................................................
Previous treatment for peri-implantitis Yes ☐ No ☐

**I/O CLINICAL EXAMINATION:**

General O.H.: Healthy ☐ Gingivitis ☐ Periodontitis ☐

\[
B
\]

Probing Pocket Depth

\[
M \quad D
\]

*bleeding on probing*

**Bone Loss from abutment/ implant junction**

Mesial = Distal =

Picture of radiograph Yes ☐ No ☐
Intra oral picture Yes ☐ No ☐
### Appendix 4 Quantitative Culture Data

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aerobic Incubation (5 days)</th>
<th>Anaerobic Incubation (10 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (p)</td>
<td>$6.8 \times 10^5$</td>
<td>$2.5 \times 10^6$</td>
</tr>
<tr>
<td>2 (h)</td>
<td>$3.2 \times 10^3$</td>
<td>$3 \times 10^5$</td>
</tr>
<tr>
<td>3 (h)</td>
<td>$8.8 \times 10^5$</td>
<td>$1.93 \times 10^6$</td>
</tr>
<tr>
<td>4 (p)</td>
<td>$2.1 \times 10^5$</td>
<td>$6.0 \times 10^6$</td>
</tr>
<tr>
<td>5 (h)</td>
<td>$5.5 \times 10^5$</td>
<td>$3.9 \times 10^6$</td>
</tr>
<tr>
<td>6 (p)</td>
<td>$2.71 \times 10^5$</td>
<td>$2.55 \times 10^6$</td>
</tr>
<tr>
<td>7 (h)</td>
<td>$5.8 \times 10^5$</td>
<td>$2.1 \times 10^6$</td>
</tr>
<tr>
<td>8 (p)</td>
<td>$5.5 \times 10^4$</td>
<td>$1.02 \times 10^7$</td>
</tr>
<tr>
<td>9 (h)</td>
<td>$1.18 \times 10^6$</td>
<td>$1.6 \times 10^6$</td>
</tr>
<tr>
<td>10 (h)</td>
<td>$2.5 \times 10^5$</td>
<td>$3.6 \times 10^5$</td>
</tr>
<tr>
<td>11 (p)</td>
<td>$2 \times 10^6$</td>
<td>$9.6 \times 10^6$</td>
</tr>
<tr>
<td>12 (p)</td>
<td>$8.3 \times 10^4$</td>
<td>$2.8 \times 10^5$</td>
</tr>
<tr>
<td>13 (p)</td>
<td>$6.7 \times 10^5$</td>
<td>$4.7 \times 10^6$</td>
</tr>
<tr>
<td>14 (p)</td>
<td>$2.1 \times 10^6$</td>
<td>$9.3 \times 10^6$</td>
</tr>
<tr>
<td>15 (h)</td>
<td>$7.9 \times 10^5$</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td>16 (h)</td>
<td>$8.5 \times 10^6$</td>
<td>$2.77 \times 10^7$</td>
</tr>
<tr>
<td>17 (p)</td>
<td>$2.8 \times 10^4$</td>
<td>$1.96 \times 10^7$</td>
</tr>
<tr>
<td>18 (h)</td>
<td>$8.5 \times 10^6$</td>
<td>$1.57 \times 10^7$</td>
</tr>
<tr>
<td>19 (h)</td>
<td>$1 \times 10^6$</td>
<td>$3.4 \times 10^6$</td>
</tr>
<tr>
<td>20 (p)</td>
<td>$1.2 \times 10^8$</td>
<td>$1.4 \times 10^8$</td>
</tr>
<tr>
<td>21 (p)</td>
<td>$1.6 \times 10^6$</td>
<td>$1.94 \times 10^8$</td>
</tr>
</tbody>
</table>

Table 28 CFU/ml from samples cultured aerobically for 5 days and anaerobically for 10 days (h), healthy (p), peri-implantitis
APPENDIX 5 PILOT TRIALS FOR BIOFILM FORMATION

The aim of these was to establish a method by which a consistent and repeatable biofilm formed of representative organisms could be grown together in vitro.

1) S. constellatus and P. gingivalis biofilms

From the preliminary data that was available from the 7 samples, it was decided that S. constellatus and P. gingivalis, would be appropriate representatives for Gram positive facultative anaerobic cocci and Gram negative strictly anaerobic rods respectively, especially that they have been associated with periodontal infections in other studies.

The method was slightly different than that described in chapter 3, and is described briefly in the following section.

STRAINS AND INOCULUM PREPARATION

S. constellatus and P. gingivalis were obtained from the frozen preserves from sample number 1. They were grown anaerobically on FAA supplemented with 5% (v/v) defibrinated horse blood for 2 days. A loopful of each bacterial strain was inoculated into 3 ml of sterilized mFUM containing 0.2% glucose and further incubated anaerobically at 37°C.

After 24 hours, 100 µl of the pre-cultures were drawn into a 96-well microtitre tray and the O.D. was measured at a wavelength of 540 nm. Bacterial suspensions were adjusted for an O.D. of 0.5 ± 0.05 (seed cultures).

PREPARATION OF BIOFILMS

Hydroxyapatite discs (HA discs), round in shape (9.5 mm and 1.5 mm in thickness) were used as a substrate for biofilm formation after being autoclaved (121°C for 20 minutes). The discs were then placed in a 24-well polystyrene cell culture plate and pre-incubated with 1ml of mFUM to precondition the discs. 100 µl of seed cultures were added to the discs with the mFUM. The growth medium was replenished every 24 hours by aspirating the spent medium from the wells and the addition of fresh medium, with the plates still in the anaerobic incubator. This was repeated 7 times (i.e. for 1 week) after which the plates were taken out.
Harvesting of the Biofilm

The HA discs were dip-washed twice and swirled gently in sterilized PBS to remove loosely adherent bacteria. Each disc was then transferred to a sterile 7ml bijou containing 1ml of FAB and six glass beads and was vortexed vigorously for 30 seconds to harvest adherent bacteria, after which the suspension was serially diluted (10^0 - 10^5). 100 μl of each diluted sample was plated on FAA (in duplicates) and the plates were incubated anaerobically at 37°C. After 10 days, the numbers of CFUs were counted and differentiation of the species was achieved by observation of colonial morphology and by Gram staining.

Staining, imaging and analysis of the biofilm was carried out as described in chapter 3.

Results and Conclusions of the Pilot Trials

After 10 days of incubation, only *S. constellatus* could be seen on the FAA plates, but *P. gingivalis* could not be detected. The effects of interactions of different species in dental plaque biofilms have been described to range from mutualistic to antagonistic. One possibility was that *S. constellatus* inhibited *P. gingivalis* and that would be quite similar to results found by Christopher et al. (Christopher et al., 2010), who reported that out of 79 Streptococcal strains, 61 resulted in the lysis of *P. gingivalis*, 17 had no effect, whilst one which was *S. intermedius* completely abolished biofilm formation by *P. gingivalis* *in vitro*. Another possibility was that *P. gingivalis* being an acid-sensitive species, sensitive to pH levels below 6.5 (Kuramitsu et al., 2007) could have been affected by the acid metabolic products (secondary metabolites) produced by *S. constellatus*.

Thus, the following modifications to the first biofilm method were attempted:

- Adding 100 μl of the inoculum containing *P. gingivalis* to the discs first. 100 μl of *S. constellatus* was then added on the fourth day, thus permitting sufficient growth of the *P. gingivalis* beforehand. The biofilm was grown for a further 3 days (i.e. a total of 7 days). The medium was changed every day as described before.
• Reducing the glucose level in the mFUM to 0.1 and 0.05% instead of 0.2%. The objective was to slow down the growth rate of *S. constellatus*, but not the *P. gingivalis*.

• After dip-washing the discs in PBS, each disc was placed in a sterile plastic petri dish. Half of the disc was scraped off using a number 15 scalpel blade on a bard-parker handle. The biofilm collected was transferred to a bijou containing glass beads and FAB by vigorously agitating the tip of the scalpel in the solution. Samples were serially diluted and plated out, inspected and counted as previously described. The other half of the disc was stained and analyzed using a CLSM.

Although, *P. gingivalis* was observed in some biofilms and these modifications enabled both species to grow together, results were inconsistent and unpredictable as can be seen in the following tables (Tables 29 – 31). Both organisms were observed only on a few discs and dilutions.

<table>
<thead>
<tr>
<th>Disc 2</th>
<th><em>S. constellatus</em></th>
<th><em>P. gingivalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution 3</td>
<td>14</td>
<td>37</td>
</tr>
<tr>
<td>Dilution 4</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

*Table 29 Biofilm CFU/ml counts grown on discs in mFUM first with 0.2% glucose replaced to mFUM with 0.05% glucose on the 4th day (i.e. on the day *S. constellatus* were added. Both organisms were detected on 1 out of 3 discs.*

<table>
<thead>
<tr>
<th>Disc 1</th>
<th><em>S. constellatus</em></th>
<th><em>P. gingivalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution 3 (x 10^4)</td>
<td>37</td>
<td>22</td>
</tr>
<tr>
<td>Dilution 4 (x 10^5)</td>
<td>33</td>
<td>3</td>
</tr>
<tr>
<td>Disc 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution 4 (x 10^5)</td>
<td>9</td>
<td>200</td>
</tr>
<tr>
<td>Dilution 5 (x 10^6)</td>
<td>2</td>
<td>21</td>
</tr>
</tbody>
</table>

*Table 30 Biofilm CFU/ml counts grown on discs in mFUM with 0.05% glucose. Both organisms were detected on 2 out of 3 discs*
<table>
<thead>
<tr>
<th>Disc</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. constellatus</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution 3 (x 10^4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disc 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution 2 (x 10^4)</td>
<td>138</td>
<td>29</td>
</tr>
<tr>
<td>Dilution 4 (x 10^5)</td>
<td>56</td>
<td>7</td>
</tr>
<tr>
<td>Disc 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution 3 (x 10^4)</td>
<td>42</td>
<td>11</td>
</tr>
<tr>
<td>Dilution 4 (x 10^5)</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Dilution 5 (x 10^6)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Disc 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution 4 (x 10^5)</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>Dilution 5 (x 10^6)</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Disc 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution 4 (x 10^5)</td>
<td>31</td>
<td>91</td>
</tr>
<tr>
<td>Dilution 5 (x 10^6)</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 31 Biofilm CFU/ml counts grown on discs in mFUM with 0.1% glucose. Both organisms were detected on 5 out of 6 discs.

The pH levels were measured daily before each media change. There was no significant drop in pH over the 7 days of biofilm formation (mean pH = 7.1 ± 0.28, range = 6.49 – 7.37, n = 42).

A different experiment was set up in which the amount of P. gingivalis added to begin with was doubled (200 µl) followed by S. constellatus (100 µl) on the fourth day, but the same inconsistent biofilms were formed. Pre-incubation using saliva + serum showed no differences in biofilm formation compared to using serum alone (data not shown).
2) *S. gordonii, F. nucleatum and P. gingivalis biofilm*

A multispecies biofilm composed of *S. gordonii, F. nucleatum and P. gingivalis* was attempted. The species were chosen because *S. gordonii* (initial colonizer) was present in all samples that contained *P. gingivalis* (late colonizer). Furthermore, some studies have shown that strains of *P. gingivalis* co-aggregate with several oral streptococci such as *S. gordonii*, and seem to augment the ability of each other to colonize into biofilms (Cook et al., 1998, Kuboniwa et al., 2006, Slots and Gibbons, 1978). Whilst, *F. nucleatum* has been suggested to possess the ability to co-aggregate initial and late colonizers, acting as a “bridge” between species and was chosen for this reason.

The mFUM media used contained 0.1% glucose and pH levels were measured daily. There was no significant drop in pH over the 7 days of biofilm formation (mean pH was 7.35 ± 0.28, range was 7.01 – 7.88, n=36). Viability of organisms was tested before the biofilm experiment, by plating 100 µl of bacterial suspension. Half of the disc was plated on FAA to determine numbers of CFUs and the other half was analyzed using CLSM.

Results on HA discs are shown in Tables 32 – 34.

<table>
<thead>
<tr>
<th>Disc</th>
<th><em>S. gordonii</em></th>
<th><em>F. nucleatum</em></th>
<th><em>P. gingivalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63 x 10⁵</td>
<td>8 x 10⁵</td>
<td>N.D.</td>
</tr>
<tr>
<td>2</td>
<td>62 x 10⁵</td>
<td>6.5 x 10⁵</td>
<td>N.D.</td>
</tr>
<tr>
<td>3</td>
<td>22 x 10⁵</td>
<td>5 x 10⁵</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Table 32 Inoculum 1 - Mean CFU/ml counts on HA discs. *P. gingivalis* was undetected. 100 µl of each *S. gordonii, F. nucleatum, P. gingivalis* was added on day 1. Biofilms were grown for 7 days. (N.D., not detected)
### Table 33 Inoculum 2 - Mean CFU/ml counts on HA discs. *P. gingivalis* was detected on 2 out of 3 discs. 100 µl of *S. gordonii, F. nucleatum* and *P. gingivalis* were added on day 1. Biofilms were grown for 7 days.

<table>
<thead>
<tr>
<th>Disc</th>
<th><em>S. gordonii</em></th>
<th><em>F. nucleatum</em></th>
<th><em>P. gingivalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>59 x 10⁴</td>
<td>63 x 10⁴</td>
<td>0.03 x 10⁴</td>
</tr>
<tr>
<td>5</td>
<td>105 x 10⁴</td>
<td>110 x 10⁴</td>
<td>0.2 x 10⁴</td>
</tr>
<tr>
<td>6</td>
<td>5.5 x 10⁴</td>
<td>13 x 10⁴</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

### Table 34 Inoculum 3 - Mean CFU/ml counts on HA discs. 100 µl of *P. gingivalis* was added on the 1st day, then 100 µl of *S. gordonii* and 100 µl of *F. nucleatum* were added on the 4th day. Biofilms were grown for 7 days. *P. gingivalis* was detected on 2 out of 3 discs.

<table>
<thead>
<tr>
<th>Disc</th>
<th><em>S. gordonii</em></th>
<th><em>F. nucleatum</em></th>
<th><em>P. gingivalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>52 x 10⁵</td>
<td>11 x 10⁵</td>
<td>N.D.</td>
</tr>
<tr>
<td>8</td>
<td>11.5 x 10⁵</td>
<td>5.5 x 10⁵</td>
<td>2 x 10⁵</td>
</tr>
<tr>
<td>9</td>
<td>5 x 10⁵</td>
<td>7 x 10⁵</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Minimal growth was still observed for *P. gingivalis* and colonies were too few to enumerate. The conclusion from these trials was to use a 2 species biofilm composed of *S. gordonii* and *F. nucleatum* and to dismiss *P. gingivalis*. 

---

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3) *S. gordonii* and *F. nucleatum* biofilms grown on titanium discs

The 2-species biofilm was grown on 4 different titanium implant surfaces (described in chapter 3). The biofilm was grown for 7 days and the experiment was repeated 10 times (i.e. n = 40). Mean CFU/ml counts from duplicate FAA plates are shown in Table 35 and CLSM representative images are shown in Figure 36.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Disc type</th>
<th>7676</th>
<th>7873</th>
<th>7874</th>
<th>6905</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S.g.</td>
<td>F.n.</td>
<td>S.g.</td>
<td>F.n.</td>
<td>S.g.</td>
</tr>
<tr>
<td>1</td>
<td>350</td>
<td>60</td>
<td>147</td>
<td>1.8</td>
<td>2320</td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>0.5</td>
<td>1690</td>
<td>110</td>
<td>63</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
<td>0.15</td>
<td>139</td>
<td>11</td>
<td>460</td>
</tr>
<tr>
<td>4</td>
<td>2580</td>
<td>75</td>
<td>70</td>
<td>8.5</td>
<td>126</td>
</tr>
<tr>
<td>5</td>
<td>905</td>
<td>50</td>
<td>95</td>
<td>20</td>
<td>44</td>
</tr>
<tr>
<td>6</td>
<td>340</td>
<td>50</td>
<td>15</td>
<td>2</td>
<td>2325</td>
</tr>
<tr>
<td>7</td>
<td>63</td>
<td>0.35</td>
<td>1690</td>
<td>110</td>
<td>630</td>
</tr>
<tr>
<td>8</td>
<td>6.9</td>
<td>0.15</td>
<td>139</td>
<td>11</td>
<td>460</td>
</tr>
<tr>
<td>9</td>
<td>2580</td>
<td>75</td>
<td>70</td>
<td>8.5</td>
<td>126</td>
</tr>
<tr>
<td>10</td>
<td>905</td>
<td>50</td>
<td>950</td>
<td>20</td>
<td>44</td>
</tr>
</tbody>
</table>

Table 35 CFU/ml x 10^2 after 1 week *in vitro* *S. gordonii* (S.g.) and *F. nucleatum* (F.n.) biofilm growth.
Table 36 CLSM images of a 2 species biofilm composed of *S. gordonii* and *F. nucleatum*, stained using Live/Dead stain. The green channel represents the live cells, while the red represents the dead cells. The characteristic spindle shape for *F. nucleatum* and cocci for *S. gordonii* can be observed.

A successful biofilm composed of both species was established. However, after careful evaluation of the aims of the study and the experience gained from the previous pilot trials, it was decided to revert to a single species biofilm. The researchers did not feel that the addition of another species would add any benefits, but may rather hinder interpretation of results. Furthermore, a 2-species biofilm was no closer to the clinical situation than a single species biofilm.

To reiterate, other studies have shown very different bacterial adhesion when a 2 species (*S. sanguinis* + *A. naeslundii*) and a 3 species (*S. sanguinis* + *A. naeslundii* + *L. salivarius*) biofilm were grown *in vitro* (Frojd et al., 2011a). Conversely biofilm formation and virulence expression by *S. mutans* were altered when grown in a dual-species model (Wen et al., 2010), whereas *V. dispar* seemed to have profited from the lactic acid produced by the streptococci and continued to grow increasingly up to the 16.5 hours of the experiment, whereas others only showed minimal increase or remained stagnant (Schmidlin et al., 2013). These studies emphasize the fact that in addition to the characteristics of the surface, the model bacteria used will influence the biofilm formation on titanium surfaces *in vitro*.
(Frojd et al., 2011a). In conclusion, it was decided that it was best to keep confounding variables to a minimum.
APPENDIX 6 MANUFACTURER DATA

5.7.4 ARITHMETIC MEAN DEVIATION OF THE SURFACES (Sa, µm)

<table>
<thead>
<tr>
<th>Roughness Parameter</th>
<th>6905</th>
<th>7874</th>
<th>7873</th>
<th>7676</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sa (µm)</td>
<td>0.8 ± 0.2</td>
<td>1.5 ± 0.07</td>
<td>0.16 ± 0.15</td>
<td>0.2 ± 0.05</td>
</tr>
</tbody>
</table>

Table 37 Arithmetic Mean Deviation of the surfaces (Sa, µm)

5.7.5 PRE-STERILIZATION CONTAMINATION TESTING AND POST-STERILIZATION ENDOTOXIN TESTING

<table>
<thead>
<tr>
<th>Surface reference number</th>
<th>Pre-sterile</th>
<th>Sterile (endotoxin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6905 TiOblast</td>
<td>0 (no bacterial colonies)</td>
<td>&lt;0.5EU /ml</td>
</tr>
<tr>
<td>7676 Test machined</td>
<td>0 (no bacterial colonies)</td>
<td>&lt;0.5EU /ml</td>
</tr>
<tr>
<td>7873 Test Ti Gr.5</td>
<td>0 (no bacterial colonies)</td>
<td>&lt;0.5EU /ml</td>
</tr>
<tr>
<td>7874 Test TiOblast + HF</td>
<td>0 (no bacterial colonies)</td>
<td>&lt;0.5EU /ml</td>
</tr>
</tbody>
</table>

Table 38 Endotoxin testing of discs - The accepted endotoxin value is 0.5EU /ml and all discs had acceptable results.
5.7.6 XPS

The elemental composition of surface 7874 is shown in Table 39 in atomic percent and indicates the presence of fluoride on these surfaces.

<table>
<thead>
<tr>
<th>Surface Ref. Nr</th>
<th>Sample</th>
<th>Position</th>
<th>C1s</th>
<th>N1s</th>
<th>O1s</th>
<th>F1s</th>
<th>Si2p</th>
<th>Ti2p</th>
<th>Fe2p</th>
</tr>
</thead>
<tbody>
<tr>
<td>7874 1</td>
<td>130415:1</td>
<td>Centre</td>
<td>20.1</td>
<td>0.8</td>
<td>55.5</td>
<td>0.5</td>
<td>0.3</td>
<td>22.9</td>
<td>-</td>
</tr>
<tr>
<td>7874 1</td>
<td>130415:1</td>
<td>Side 1</td>
<td>25.3</td>
<td>0.9</td>
<td>52.6</td>
<td>0.6</td>
<td>0.4</td>
<td>20.2</td>
<td>-</td>
</tr>
<tr>
<td>7874 1</td>
<td>130415:1</td>
<td>Side 2</td>
<td>25.6</td>
<td>0.9</td>
<td>52.5</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>21.0</td>
<td>-</td>
</tr>
<tr>
<td>7874 2</td>
<td>130415:2</td>
<td>Centre</td>
<td>23.0</td>
<td>0.7</td>
<td>54.9</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>21.5</td>
<td>-</td>
</tr>
<tr>
<td>7874 2</td>
<td>130415:2</td>
<td>Side 1</td>
<td>22.5</td>
<td>0.6</td>
<td>55.1</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>21.7</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>7874 2</td>
<td>130415:2</td>
<td>Side 2</td>
<td>20.4</td>
<td>0.5</td>
<td>56.7</td>
<td>&lt;0.3</td>
<td>0.3</td>
<td>22.1</td>
<td>-</td>
</tr>
<tr>
<td>7874 3</td>
<td>130415:3</td>
<td>Centre</td>
<td>19.6</td>
<td>0.5</td>
<td>57.3</td>
<td>0.3</td>
<td>&lt;0.3</td>
<td>22.2</td>
<td>-</td>
</tr>
<tr>
<td>7874 3</td>
<td>130415:3</td>
<td>Side 1</td>
<td>22.4</td>
<td>0.9</td>
<td>54.5</td>
<td>0.5</td>
<td>&lt;0.3</td>
<td>21.7</td>
<td>-</td>
</tr>
<tr>
<td>7874 3</td>
<td>130415:3</td>
<td>Side 2</td>
<td>20.0</td>
<td>0.6</td>
<td>57.6</td>
<td>0.5</td>
<td>&lt;0.3</td>
<td>21.4</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 39 Elemental composition of surface 7874 indicating traces of fluoride