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An assessment of early colonisation of implant-abutment metal surfaces by single species and co-cultured bacterial periodontal pathogens

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Abstract

Objective

Numerous studies have proposed that smooth metal surfaces reduce initial bacterial attachment in the establishment of an early biofilm formation. However, these studies have largely examined single bacterial species, which are not always relevant as pathogens identified as initiators of inflammatory peri-implantitis. This study investigated the adherence of four periodontally-relevant bacterial species to implant and abutment surfaces in current clinical use.

Methods

Discs of polished cobalt chromium (CoCr-polished) and milled titanium (Ti-milled), representing two clinically relevant surfaces, were prepared and surfaces were characterised. Bacterial species *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Aggergatibacter actinomycetemcomitans* were cultured to mid-log or stationary growth phase. Co-cultures of *P. gingivalis*, *F. nucleatum* and *P. gingivalis*, *F. nucleatum*, *Pr. intermedia* were similarly prepared. Bacteria were inoculated onto discs for 2 hours, stained with a live/dead fluorescent stain and percentage bacterial coverage was calculated by confocal microscopy and image analysis.

Results

CoCr-polished discs had smooth surfaces with gentle valley structures, whilst Ti-milled discs had sharp edged peaks. Both discs demonstrated a partial wetting ability capable of initiating bacterial adhesion. *P. gingivalis*, *F. nucleatum* and co-cultures, at both mid-log and stationary concentrations, demonstrated equally high coverage of both the smooth CoCr-polished and the rougher Ti-milled metal surfaces. *Pr. intermedia* and *A. actinomycetemcomitans* demonstrated lower surface coverage which was slightly higher for Ti-milled.

Conclusion

Variability was noted in the adherence potential for the respective periodontal pathogens examined. Particularly high adherence was noted for *P. gingivalis* and *F. nucleatum*, despite the manufacture of a smooth surface.

Clinical significance

Both surfaces studied may be used at implant-abutment junctions and both possess an ability to establish a bacterial biofilm containing a periodontally-relevant species. These surfaces are thus able to facilitate the apical migration of bacteria associated with peri-implantitis.

Running Head: Bacterial adherence to smooth metal surfaces

Key words: periodontal pathogens, bacterial adherence, cobalt chromium, titanium, smooth surfaces, milled surfaces

1. Introduction

Peri-implant disease is regarded as an infectious disease, whereby oral pathogens, usually gram-negative micro-organisms, initiate host inflammatory responses [1,2]. Peri-implant mucositis describes the inflammation of the soft tissues surrounding the implant that can progress to peri-implantitis and involves apical migration of the bacteria, effecting inflammatory destruction of the supporting bone and potential loss of the implant [3]. Approximately 1000 bacterial species have been identified in the oral cavity, of which approximately 10 species have been implicated as periodonto-pathogens, that have a strong association as initiators of the host response leading to peri-implant disease [4,5]. Of these periodonto-pathogens significant research literature is available to support pathogenic roles for opportunistic bacteria *Aggergatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Porphyromonas gingivalis* [reviewed by 4]. Despite *P. gingivalis* being identified as a low abundance biofilm species, evidence suggests that it can orchestrate inflammatory destruction [6] and often co-aggregates together with *Pr. intermedia* [7]. The adherence of periodonto-pathogens to dental implants or abutments exposed to the oral cavity can lead to transfer of bacteria to the trans-gingival region around the implant / abutment leading to inflammation of the soft tissues. The elimination of biofilms from implant surfaces is thus a significant objective in preventing peri-implant disease.

Virulence factors, such as lipopolysaccharides and other bacterial surface proteins released from the bacteria, are now well regarded as initiators of inflammatory destruction via activation of the innate and acquired immune response [8,9,10]. Continued burden of bacterial virulence factors can trigger an excessive immune response and an imbalanced homeostatic response of the resident connective tissue cells, leading to net activation of tissue degradation pathways, such as osteoclast activation and destruction of the supporting bone.

In addition, peri-implantitis progression is determined by the nature of the host immune response to these specific microorganisms within the biofilm. In susceptible individuals, inflammatory destruction is exasperated by genetic and / or systemic factors such as neutrophil dysfunction, osteoporosis, diabetes and smoking. Environmental factors, such as macro / micro occlusal stresses placed on the supporting bone can also lead to inappropriate immune responses, particularly if long-term incorrect loading of the implant occurs, which may increase susceptibility to inflammatory bone loss. These conditions can only be resolved via eradication of the bacterial pathogens as initiators of the host response [3]. If implants have been used as replacements for teeth lost as a consequence of periodontal disease, then similar compromising factors would be expected to be present in allowing the progression to peri-implantitis.

The design and production of implants and abutments follow BS EN ISO standards. Whilst genotoxicity studies affecting bacterial mutations are performed, bacterial adherence is not currently a requirement and thus no standard GLP protocol has been established. Adherence of bacteria is dependent upon the physiochemical nature of the cell wall, fimbriae attached to the bacteria and the surface characteristics of the metal surface. Previous studies have indicated that bacterial adherence can be affected by crystallinity, depth of the oxide layer and surface roughness [11]. Published data suggests that cobalt chromium metal surfaces demonstrate reduced bacterial adherence compared with titanium, although this is not significant for all studies [12,13,14]. With regards to surface roughness, studies have suggested higher bacterial adherence due to irregularities on polymeric surfaces compared to ultrasmooth surfaces [15,16]. However, for the majority of these studies the adherence of just one bacterial species was investigated, such as *Staphylococcus epidermidis* [13,17], *Streptococcus mutans* [18], or *Staphylococccus aureus* [19] all of which are species associated with plaque formation and development of a caries lesion rather than peri-

implantitis. No studies have investigated the adherence of the different periodontally relevant bacterial species which can guide bacteria to sites for the initiation of peri-implantitis. Further these studies fail to take into account that peri-implantitis is initiated following the colonisation by a polymicrobial biofilm [4,5].

The aim of this study was to investigate the adherence of populations of periodontally relevant bacterial species to an electropolished smooth cobalt chromium surface and milled titanium surface prepared as discs, representing surfaces of dental abutments and implants. Of note, bacteria adhering to the abutment surfaces may derive from free-floating rapidly growing bacteria in saliva or slow dividing bacteria living in a biofilm. The same species can have significantly different properties, recognising that bacteria existing in densely protected environments cooperate and interact in different ways to planktonic bacterial suspensions facilitated by altered quorum sensing. To investigate whether this affected adherence properties of bacteria to metal surfaces this study also looked to compare bacterial properties in log-growth stage and stationary phase of culture. The study lays the foundation for development of research protocols which is a relevant consideration in the functioning of implants and abutments within the oral cavity.

2. Materials and Methods

2.1 Growth conditions of anaerobic bacteria

The strains used in this study were *Porphyromonas gingivalis* NCTC11834, *Fusobacterium nucleatum* ATCC49256, *Prevotella intermedia* NCTC13070 and *Aggergatibacter actinomycetemcomitans* DSM8324.

P. gingivalis, *F. nucleatum* and *Pr. intermedia* were maintained on Fastidious Anaerobe Agar (FAA; Lab M Ltd., Lancashire, UK) plates containing 5% defibrinated horse blood (TCS Biosciences Ltd., Buckingham, UK) at 37°C in an anaerobic environment (80% nitrogen, 10% carbon dioxide, 10% hydrogen) in a Modular Atmosphere Controlled System. *A. actinomycetemcomitans* was maintained on FAA plates containing 5% defibrinated horse blood at 37°C in 5% CO_2 in air (Sanyo CO_2 incubator). Liquid cultures of *P. gingivalis*, *F. nucleatum* and *Pr. intermedia* were grown under anaerobic conditions at 37°C in Fastidious Anaerobe Broth (FAB; Lab M) while *A. actinomycetemcomitans* was cultured in FAB at 37°C in 5% CO_2 in air. Culture purity was determined by colony appearance on FAA, Gram staining and microscopic analysis.

2.2 Bacterial growth curves

A. actinomycetemcomitans, previously grown on FAA for 48 h in 5% CO_2 in air at 37°C, was sub-cultured into 10 ml FAB and cultured in 5% CO_2 in air at 37°C overnight without agitation. The broth culture was diluted to an initial inoculum of ~1×10^7 cells/ml and OD_{600} readings were recorded every hour for 18 h using a FLUOstar Omega plate reader with

atmospheric control unit (O_2/CO_2 Mpc GV; BMG Labtech GmbH, Ortenberg, Germany). Growth curves were plotted from three separate repeat experiments.

Pr. intermedia, F. nucleatum, P. gingivalis, all previously grown on FAA for 72 h in an anaerobic environment at 37°C, was sub-cultured into 10 ml FAB prior to overnight culture in anaerobic conditions without agitation (exception *P. gingivalis* cultured for 40 h). Culture broths were diluted to an initial inoculum of 1×10^8 cells/ml (*Pr. intermedia*), 5×10^7 cells/ml (*F. nucleatum*) or 1×10^8 cells/ml (*P. gingivalis*) and OD_{600} readings were recorded every hour for 20 h using an IMPLEN GmbH (Müchen, Germany) OD600 DiluPhotometer™. Bacterial numbers along the growth curves obtained were determined using the Miles and Misra technique [20] on FAA plates. *A. actinomycetemcomitans*, *Pr. intermedia*, *F. nucleatum* and *P. gingivalis* were cultured in FAB broth as described for determination of the bacterial growth curves and the broth was diluted to a range of optical densities which correspond to points on the growth curves. These dilutions were further serially diluted 1 in 10 in sterile PBS to form a dilution series from 10^1 to 10^8. Aliquots of these dilutions (10 µl) were inoculated onto FAA plates and cultured for 24 h (*P. gingivalis* cultured for 40 h) at the appropriate atmospheric condition before counting bacterial colonies to determine plating numbers.

2.3 Metal surfaces and hydrophobicity assessment

Metal discs were supplied by Renishaw plc as either electropolished Cobalt Chromium (CoCr-polished) or machined Titanium 6-Aluminium 4-Vanadium (Ti64) (Ti-milled).

The Ti-milled discs were prepared from Ti64 (ASTM Grade 5) bar stock on a Schaublin 102N manual lathe. The CoCr-polished discs were additively manufactured from Renishaw

CoCr DG1™ metal powder on the Renishaw AM250 via the process of Selective Laser Melting (SLM). Build supports were manually removed and the disc surfaces dressed using a handheld rotary tool and tungsten carbide burr. The discs then underwent an electropolishing process in which they were connected to the positive side of a power supply (hence acting as the anode), submerged in an electrolyte along with a conformal cathode, and a voltage was applied in order to give a predetermined current density over the discs for a set time period. The resulting electrochemical reaction caused material to be preferentially removed from surface maxima on the disc, giving a polished appearance.

Surfaces of the discs were imaged using Leica TCS SP2 AOBS spectral confocal microscope with a 20x objective lens. A reflectance signal was generated using a 488nm laser, with the detector sampling reflected light from the surface of the discs. Optical sections were taken at 0.6 micron intervals over the entire surface of the disc and Z-stack sections were reconstructed into single maximal intensity projections. Surface roughness measurements of discs were taken using a Suftest SV-2000 profilometer (Mitutoyo, Hampshire, UK) across a distance of 25μm at a speed of 0.1mm/s (n=6). Mean roughness values (Ra) and maximum heights (Ry) were calculated by Surfpak-SV software (Mitutoyo, Hampshire, UK) and surface profiles combined from 6 individual measurements. Hydrophobicity was measured for each surface (n=6) using a Dynamic Contact Angle Analyser 312 (Cahn Instruments Inc., Thermo Electron Corporation), using deionised distilled water as the wetting medium. Prior to each measurement discs were placed in a drying cabinet at 95°C overnight. Each disc was repeatedly assessed on 5 different days where environmental temperature recording were equivalent. Advancing and receding contact angles were calculated using the least squares analysis generated by Cahn Application Software. Average advancing contact angles and standard deviations were calculated.

2.4 Bacterial adherence studies

Bacterial adherence studies were performed for each surface at bacterial concentrations representative of the exponential mid-log growth phase and the stationary phase. Briefly, the respective bacterial cultures were prepared in FAB as described above and then diluted in further broth to give the following OD_{600}: *P. gingivalis* mid-log, 0.75, stationary 1.3; *F. nucleatum* mid-log 0.35, stationary 0.75; *Pr. intermedia* mid-log 0.75, stationary 1.6; *A. actinomycetemcomitans* mid-log 0.13, stationary 0.19. In addition the adherence of co-cultures for *F. nucleatum* / *P. gingivalis*, or *F. nucleatum* / *P. gingivalis* / *Pr. intermedia*, were examined. Bacteria were prepared to OD equivalent to either their mid-log or stationary concentration and then combined.

Metal discs (CoCr-polished and Ti-milled) were sterilised by autoclaving at 121°C for 15 min and dried prior to use. Discs were placed into wells of a 24 well plate and 2 ml bacterial suspension was pipetted onto each disc (FAB culture broth served as a negative control) and culture was continued for 2 h under the appropriate growth conditions described above. Discs were removed and washed with 1 ml PBS in a fresh well, with very gentle agitation for 1min. Bacteria adherent to the disc was stained with 10 µl of LIVE/DEAD® BacLight™ Bacterial Viability stain (Molecular Probes; as per manufacturer's instructions) for 30 min prior to examination by confocal laser scanning fluorescence microscopy (Leica SP5 Confocal Microscope) at x40 magnification. Five random images were obtained for each sample using LAS-AF software, version 2.6.0.7266. All digital images were examined to determine the percentage area of fluorescence (representing the bacterial coverage) on the disc surface using ImageJ. Thresholds were applied to the images and converted into binary black and white images to allow for quantification of bacterial coverage. Discs were mechanically cleaned using a toothbrush and re-autoclaved. Removal of bacteria was

confirmed by re-examination under confocal microscopy. The bacterial adherence assay was repeated on the same batch of discs on one further occasion.

2.5 Statistical analysis

For each bacterial species (grown to either mid-log or stationary phase) and each surface analysed the mean and standard error of the mean (SEM) was determined. Statistical significance was assessed using an ANOVA test with Tukey-Kramer correction test for multiple comparisons (Instat, GraphPad Software, San Diego, USA). Growth curve raw data were graphically depicted using BMG Labtech Mars data analysis software.

3. Results

3.1 Surface characteristics of disc surfaces

All surfaces had a similar partial wetting ability. The average advancing contact angles, for n=6, were calculated as 78.39 ± 1.735 for the CoCr-polished surfaces and 83.176 ± 2.531 for Ti-milled surfaces. Topographical images and surface roughness profiles ~~are~~ for the CoCr-polished and Ti-milled surfaces are shown in Fig. 1. The Ti-milled surfaces demonstrated a sharp edged concentric circular pattern consistent with the milling procedure used in their preparation. The CoCr-polished surfaces had a more irregular pattern with the appearance of smooth and shallow edged grooves. Surface profilometry data (Fig. 1B) indicated that Ti-milled had significantly rougher surface (average surface roughness Ra = 0.179±0.055) with larger peaks and valleys (maximum peak-to-valley height, Ry = 0.997±0.447) compared to

the CoCr-polished surface, (Ra = 0.028±0.0067; Ry = 0.165±0.0.034), with statistical analysis indicating $p<0.0001$.

3.2 Determination of growth and stationary phase of bacterial species

Fig. 2 shows the growth curves obtained for each of the bacterial species examined. For all graphs the right hand axis indicates true bacterial count expressed as bacterial colony forming units/ml (CFUs/ml) determined using the Miles and Misra method. From these graphs the bacterial concentration / OD_{600} representative of the mid-log growth phase when the bacteria are actively proliferating and the stationary growth phase when proliferation has ceased were determined (indicated on the graph). Bacterial inoculating concentrations for mid-log and stationary growth phase are reported in Fig. 2.

3.3 Bacterial adherence to disc surfaces

Bacteria, adjusted to a cell concentration equivalent to mid-log active growth phase or the stationary growth phase (figure 2), were inoculated onto the metal surfaces and adherence to the surfaces was observed after 2 h. Confocal images of fluorescently stained bacteria adherent to the surface are shown for the CoCr-polished (Figure 3) and the Ti-milled (Figure 4). Imaging procedures were conducted in aerobic conditions during which time anaerobic bacteria were noted to become non-viable. For this reason, bacteria staining for both viable fluorescently green bacteria and dead fluorescently red bacteria were counted. These images were converted into binary black and white images (shown in Figures 3 and 4) and percentage coverage was calculated using ImageJ (Figure 5A).

Considering first single species inoculations, particularly high bacterial coverage was noted for the periodontal pathogen *P. gingivalis* and *F. nucleatum* to both the CoCr-polished (Figure 3) and the Ti-milled surfaces (Figure 4). Following image analysis (Figure 5A) bacterial coverage of surfaces by *P. gingivalis* at mid-log growth phase was substantial despite bacterial concentrations (CFUs/ml) 40 times lower compared to stationary concentration (46 fold lower CFUs for *F. nucleatum* mid-log concentration compared to stationary concentration). Statistical analysis indicated that there was no difference in coverage of the CoCr-polished and the Ti-milled surfaces by the pathogen *P. gingivalis* and *F. nucleatum* when inoculated at either the mid-log or the stationary growth phase. Lower levels of bacterial coverage were observed for *Pr. Intermedia.* For bacteria in the stationary growth phase attachment was statistically significantly higher on Ti-milled compared to the polished cobalt chromium surface ($p<0.001$) but no statistically significant difference was observed comparing mid-log and stationary growth phase inoculants. Bacterial coverage of the surfaces by *A. actinomycetemcomitans* was very low, and no statistically significant differences were observed in the percentage coverage at the mid-log and stationary phase concentrations.

Statistical analysis was performed to compare bacterial coverage following inoculation at either mid-log or stationary growth phase to a particular surface. When percentage bacterial coverage / 2ml of inoculant was compared there was generally no difference for bacteria in the mid-log and stationary growth phase in coverage to the surface, despite the higher bacterial load in the stationary phase inoculant (exception was seen for *P. gingivalis* on CoCr-polished).

Inoculation of co-cultures of bacteria demonstrated differing results to those presented above. Analysis only focused on combinations of the anaerobic bacterial species, recognising *A.*

actinomycetemcomitans as an aerobic bacteria and difficulty of culture with anaerobic bacterial species. High levels of anaerobic bacterial adherence were noted on both surfaces (Figures 3 and 4). Statistical analysis of image analysis data indicated that a higher level of percentage bacterial coverage was observed for co-cultures (at mid-log and stationary phase inoculations) and triple cultures (mid-log phase only) on CoCr-polished surfaces (Figure 5A).

4. Discussion

Within this study two very contrasting metal surfaces were examined for their ability to form a bacterial biofilm consisting of periodonto-pathogens with high clinical relevance leading to the induction of peri-implantitis and thus the potential loss of an endosseous prosthesis. The highly CoCr-polished represents a surface commercially used for dental abutments with a purported reduced bacterial adherence and is compared with a contrasting Ti-milled surface, which is proposed to harbour bacteria within its roughened profile and facilitate biofilm formation. Contrary to this hypothesis, this study indicated that both the smooth CoCr-polished and the rougher Ti-milled surfaces were able to facilitate the establishment of significant coverages by the periodonto-pathogens *P. gingivalis* and *F. nucleatum* and co-cultures containing these bacterial species. Although, high adherence of *P. gingivalis* to rough Ti-milled surfaces has previously been reported [21], significantly, this study reports for the first time the differential ability of periodonto-pathogenic bacterial species to attach to

both CoCr-polished and Ti-milled, with reduced adherence levels observed for *Pr. Intermedia* and *A. actinomycetemcomitans*.

The species examined in this examination are all recognised as prime pathogens in producing virulence factors capable of inducing inflammatory destruction of the peri-implant mucosal and osseous tissues. During plaque formation, gram-positive commensals such as *Streptococcus gordonii* and related *Streptococci* and *Actinomyces* species act as initial colonizers in the formation of supragingival plaque above the gum line. Later biofilm inhabitants such as *P. gingivalis*, *F. nucleatum*, *Pr. Intermedia* and *A. actinomycetemcomitans*, are capable of binding to the antecedent organisms, which migrate under the gingival margin to form subgingival plaque with pathogenic potential, acting as initiating factors of periodontal disease or peri-implantitis. The attachment of bacteria to abutments, particularly areas bordering with the gingival tissue can act as a reservoir for directing pathogenic bacteria to the gingival sulcus from where apical migration down a root or implant can lead to inflammatory tissue destruction of the supporting boney tissues. Metal abutments and the neck of implants abutting mucosal tissue have been designed with highly polished smooth surfaces with a view to reduce bacterial adherence. However, the substantial clinical data available is conflicting which probably arises from the study of just one bacterial species, subtle differences in surface preparation and the variety of assay systems employed [15,16]. Studies have indicated that surface roughness below 300 nm Ra can still promote bacterial adherence [13] but studies have also suggested that a stronger influence to bacterial adherence is hydrophobicity [13] and bacterial adherence has been reported for titanium, cobalt chromium and stainless steel [12, 14, 22]. Within the present study, the presence of a significantly smoother surface for the polished CoCr discs was clearly established from confocal imaging and profilometry measurements. The results from our study would thus support the viewpoint that the smoothing of a surface may not reduce

the ability of bacteria to adhere to metallic surfaces. Notably both surfaces examined in this study demonstrated a remarkably high coverage for the periodonto-pathogens *P. gingivalis* and *F. nucleatum*. This would suggest a high potential for these clinically relevant bacteria to establish themselves as substantial microbial biofilms, particularly at locations where bacteria shelter from salivary sheer forces. This would eventually lead to the formation of gingival inflammation and subsequently periodontal disease / peri-implantitis.

All surfaces examined had a moderate wettability, with a slightly higher wettability for CoCr-polished surfaces. This would favour the adherence of these bacteria to each of these surfaces, with all bacteria examined in this study known to be hydrophilic and carry a negative charge [23,24]. The outer membrane of these gram-negative bacteria are complex, yet highly organised asymmetric structures, containing variable amounts of lipopolysaccharides and surface appendages such as fimbrae and conjugative pili representing rod-like arrays of protein structures. Initial biofilm formation involves the formation of weak, reversible interactions brought about by long-range van der Waals attractive forces [25]. These are subsequently stabilised by stronger ionic stereochemical interactions between the bacterial surface proteins and, within this experimental scenario, constituents of the culture broth (such as yeast extracts and small molecular weight peptones) bridging binding with the metal surface [25]. With the exception of *P. gingivalis* at mid-log growth phase concentration, for all other single species inoculations there was no statistical difference in the levels of bacterial adherence to the CoCr-polished surface and the Ti-milled surface. This would support suggestions that early adherence over the first 2 hours is largely reliant on the hydrophilic-hydrophobic properties of the surface in attracting the bacteria [13].

An important observation within this study is that high levels of bacterial coverage of the surfaces are seen by confocal microscopy following inoculation with a co-culture consisting

of two or three anaerobic bacterial species. Following inoculation with co-cultures prepared to mid-log growth phase, statistical comparison of image analysis data indicated that bacterial coverage is 0.5-2 times higher on the smooth CoCr-polished surface compared with the rougher Ti-milled surface, a feature not observed with the single inoculate cultures. This, possibly unexpected result, may reflect the co-operative influences of bacteria in forming biofilms of defined patterns of co-aggregation [25] and the subsequent adherence of these aggregates to surfaces with differing topography. Our hydrophobicity measurements indicated that both surfaces possessed similar surface free energy for the initial attraction of the bacteria. However, the sharp-edged architecture of the milled surface may result in steric hindrance for bacterial aggregates that may form and thus weaker interaction with the surface. A smoother surface may be conceived to provide a flatter surface area and more attraction points for facilitating the initial electrostatic interaction and subsequent anchorage to the surface of large bacterial aggregates. A further observation noted within this study indicated that co-cultures of *P. gingivalis/F.Nucleatum* had a significantly higher coverage on the metal surfaces compared with the triple culture preparation of *P. gingivalis/F. nucleatum/Pr. intermedia*. This again is an unexpected result since co-operative interactions have previously been identified between *P. gingivalis* and *Pr. intermedia* in forming biofilms [7], and our results would suggest co-cultures of *P. gingivalis/F. nucleatum* would also enhance adherence to the metal surfaces compared with the respective single inoculum cultures. However, low adherence was also noted for single cultures of *Pr. intermedia* to the metal surfaces and this would suggest that it has an inhibitory or repulsive effect of the bacterial aggregates for surface coverage when inoculated with other high adherent strains of *P. gingivalis/F. nucleatum.*

Through the mechanism of quorum sensing, bacterial surface architecture and surface appendages have the potential to change during bacterial growth and when growth slows in

the stationary phase. For this reason this study also provided preliminary data that investigated the adherence of the periodonto-pathogens with the metal surface when bacteria had been cultured to a mid-log and stationary growth phase concentrations. Image analysis data reflecting the percentage coverage of the surface / 2ml inoculum indicated no difference in bacteria adherence at mid-log and stationary growth phase, despite the 40-fold difference (approximate value) in bacterial concentration, indicating that adherence is not concentration dependent. Whilst not proven in this study, it could be speculated that bacteria do change their surface physiochemical characteristics dependent upon growth status, which could influence the van der Waal's forces of attraction of bacteria to a surface with bacteria in the mid-log growth phase possessing a higher affinity. This would require extensive kinetic adherence studies and analysis of zeta potentials of bacteria to confirm. However, it is clear from this study that bacteria in either mid-log stage are equally able to quickly establish a biofilm to provide similar coverage as bacteria in the stationary growth phase. It is also worth considering the clinical *in vivo* value of these results, where bacteria are passively transported to the surface of abutments and exposed cervical regions of implants via the aqueous environment of saliva. Bacterial load within the mouth will be variable, but the results of this study would also suggest that low levels of actively growing co-cultures of pathogens such as *F. nucleatum* and *P. gingivalis* would appear to possess a more active ability to adhere to metallic surfaces when compared to bacteria at higher concentrations.

In summary, this study indicates that smooth surfaces are still capable of attracting substantial levels of specific bacteria to its surface, particularly *F. nucleatum and P. gingivalis* and co-cultures consisting of these periodontal pathogens. Transient bacterial adherence to the metallic surfaces would allow for its ready transfer to below the gingival margin, commencing the process of peri-implantitis. Bacteria such as *Pr. Intermedia* and *A. actinomycetemcomitans* did indicate particularly low ability to adhere to the metal surfaces

which may inhibit the surface coverage of high adherent bacterial strains of P. gingivalis and F. nucleatum in mixed cultures. The study thus indicates the variable nature of bacterial species in adherence studies and the importance for the analysis of a range of different and relevant bacterial species, including co-cultures, in providing a better overall assessment of antibacterial properties of dental abutments and implants. This study also poses the question if altering surface roughness or the metal type or chemistry is likely to achieve the required level of elimination of bacterial adherence to dental implants and abutments, to promote stable, longer-lasting and functional fixed dental prostheses.

5. References

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Figure legends

Fig. 1: Characterisation of the surface roughness of the prepared discs. (A) Surfaces were visualised by confocal laser microscopy using reflected light mode and demonstrated contrasting smooth surface for the CoCr-polished compared with the rougher surface of the Ti-milled. (B) Surface profilometry indicated that the emergence profile for the polished Co-Cr surface is 5.8 times smoother (for both Ra and Ry) compared to the milled titanium.

Fig. 2: Growth curves for periodonto-pathogens *Pr. intermedia, P. gingivalis* and *F. Nucleatum,* grown under anaerobic conditions and *A. actinomycetemcomitans,* grown under capnophilic conditions. Bacterial CFUs were also determined from which mid-log and stationary growth phase concentrations were identified for each bacteria. These represented the concentration of bacteria inoculated onto the metal surfaces.

Fig 3: Representative confocal imaging of bacterial species, and co-culture combinations, adherent to CoCr-polished surfaces. Using Image J, confocal images were converted into the respective black and white binary image shown alongside, and percentage black was calculated to equated to percentage bacterial coverage. Average bacterial coverage was calculated from 5 random fields of view per disc, repeated on 3 separate occasions.

Fig. 4: Representative confocal imaging of bacterial species, and co-culture combinations, adherent to Ti-milled surfaces. Percentage bacterial coverage of the surface was calculated from the converted binary images and averages calculated from 5 random fields of view per disc, repeated on 3 separate occasions.

Fig: 5: Comparison and statistical analysis of average percentage bacterial coverage 2hrs following inoculation of the respective surface with 2ml of bacteria in broth at either mid-log

or stationary growth phase concentration. For bacterial species and co-cultures studied no statistical difference was observed between inoculation at the mid-log and stationary growth stage (exception *P. gingivalis/ F. nucleatum / Pr. intermedia* co-culture where higher coverage was observed to milled Ti at stationary phase). Higher adherence was observed to the CoCr-polished surface for *P. gingivalis / F. nucleatum* at both growth phase and *P. gingivalis/ F. nucleatum / Pr. intermedia* in the mid-log phase. No statistical differences in percentage bacterial coverage of CoCr-polished and Ti-milled was observed for other inoculations examined. *A. actinomycetemcomitans* and *Pr. intermedia* demonstrated low adherence to both surfaces *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 1

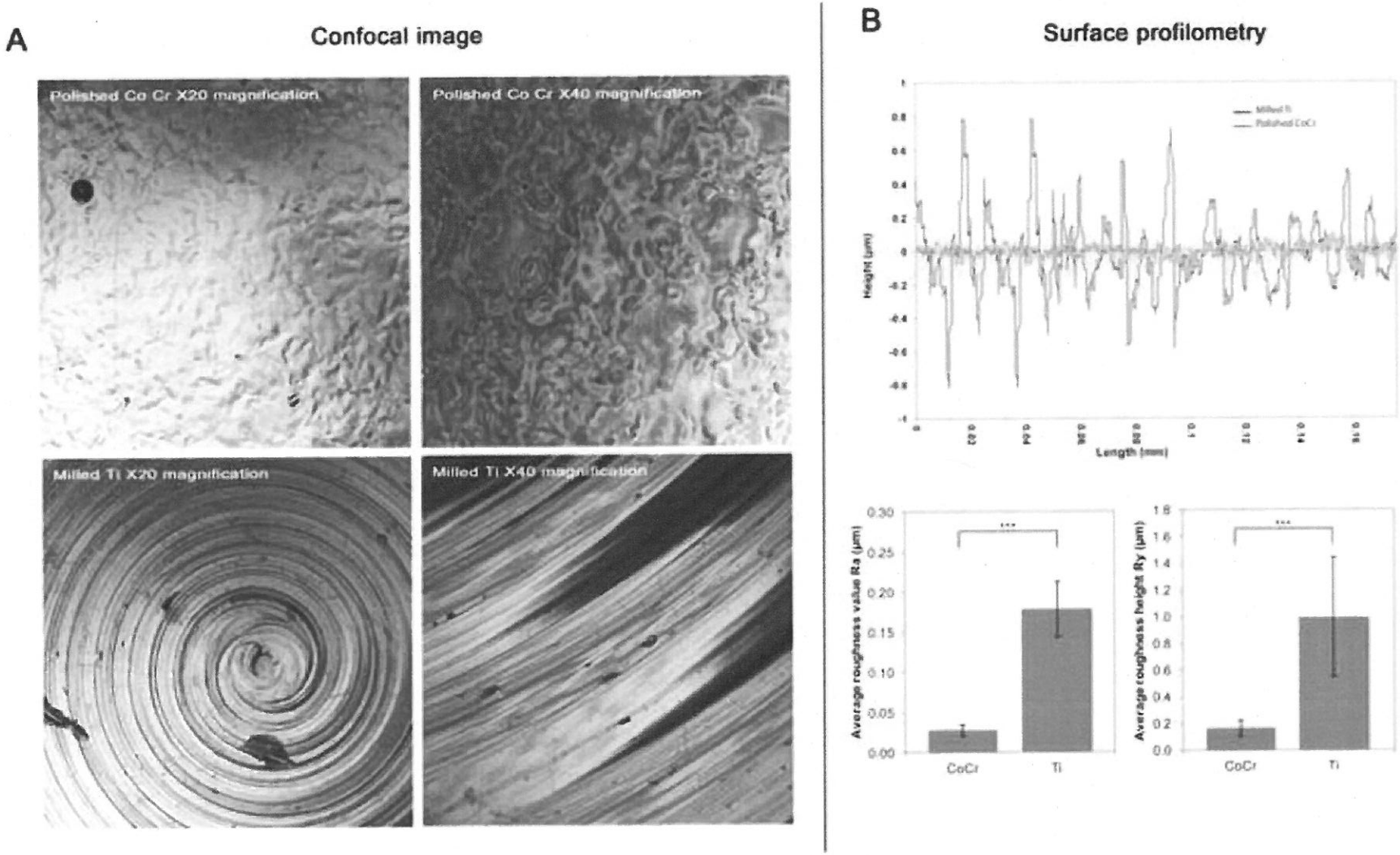

A
Confocal image
Polished Co Cr X20 magnification
Polished Co Cr X40 magnification
Milled Ti X20 magnification
Milled Ti X40 magnification
B
Surface profilometry
Milled Ti
Polished CoCr
Height (µm)
Length (mm)
Average roughness value Ra (µm)
Average roughness height Ry (µm)
***
***
CoCr
Ti
CoCr
Ti

Figure 2

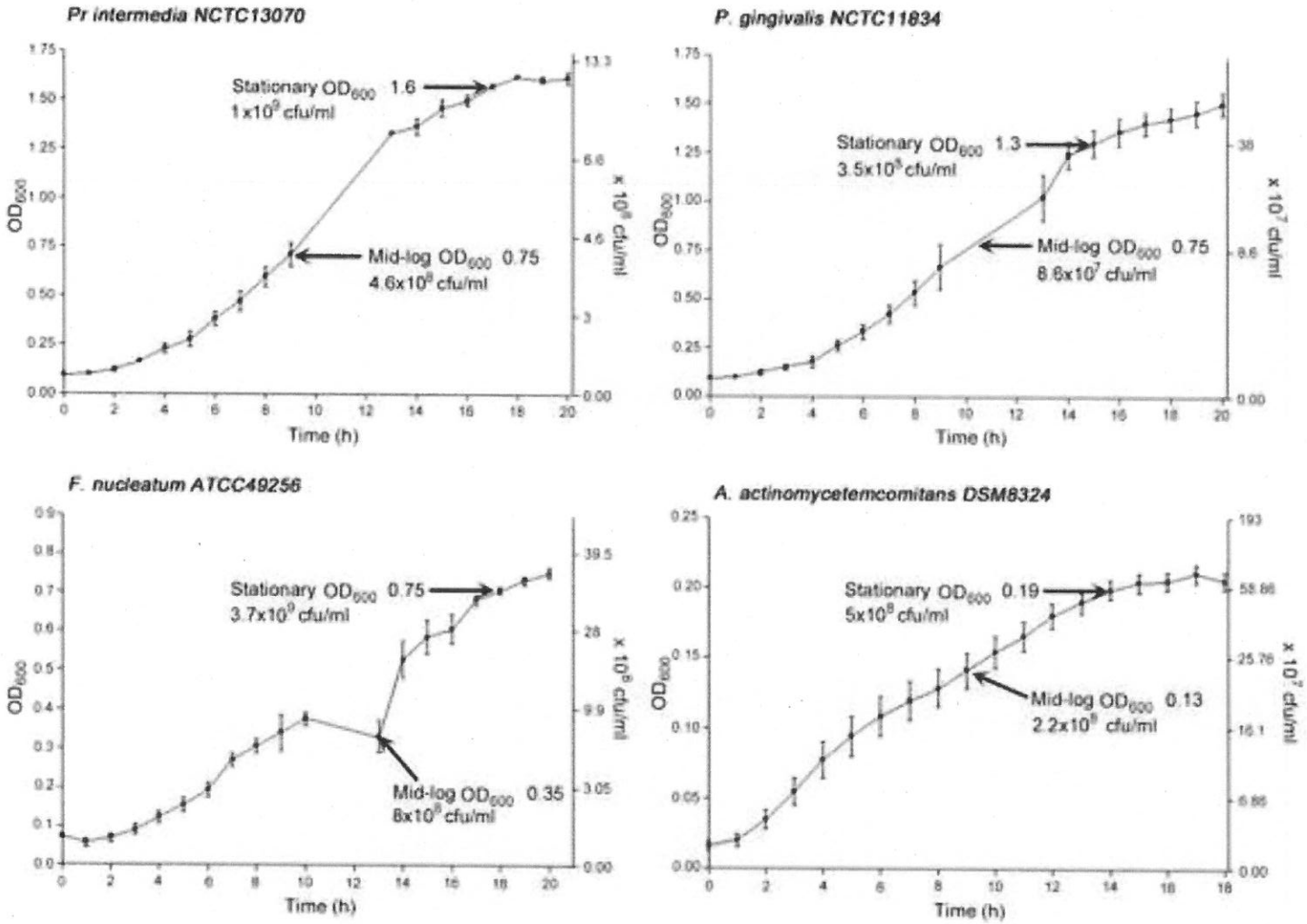

Pr intermedia NCTC13070
Stationary OD600 1.6
1x10^9 cfu/ml
Mid-log OD600 0.75
4.6x10^8 cfu/ml
OD600
x 10^8 cfu/ml
Time (h)
P. gingivalis NCTC11834
Stationary OD600 1.3
3.5x10^8 cfu/ml
Mid-log OD600 0.75
8.6x10^7 cfu/ml
OD600
x 10^7 cfu/ml
Time (h)
F. nucleatum ATCC49256
Stationary OD600 0.75
3.7x10^9 cfu/ml
Mid-log OD600 0.35
8x10^8 cfu/ml
OD600
x 10^8 cfu/ml
Time (h)
A. actinomycetemcomitans DSM8324
Stationary OD600 0.19
5x10^8 cfu/ml
Mid-log OD600 0.13
2.2x10^8 cfu/ml
OD600
x 10^7 cfu/ml
Time (h)

Figure 3

Polished Cobalt Chromium

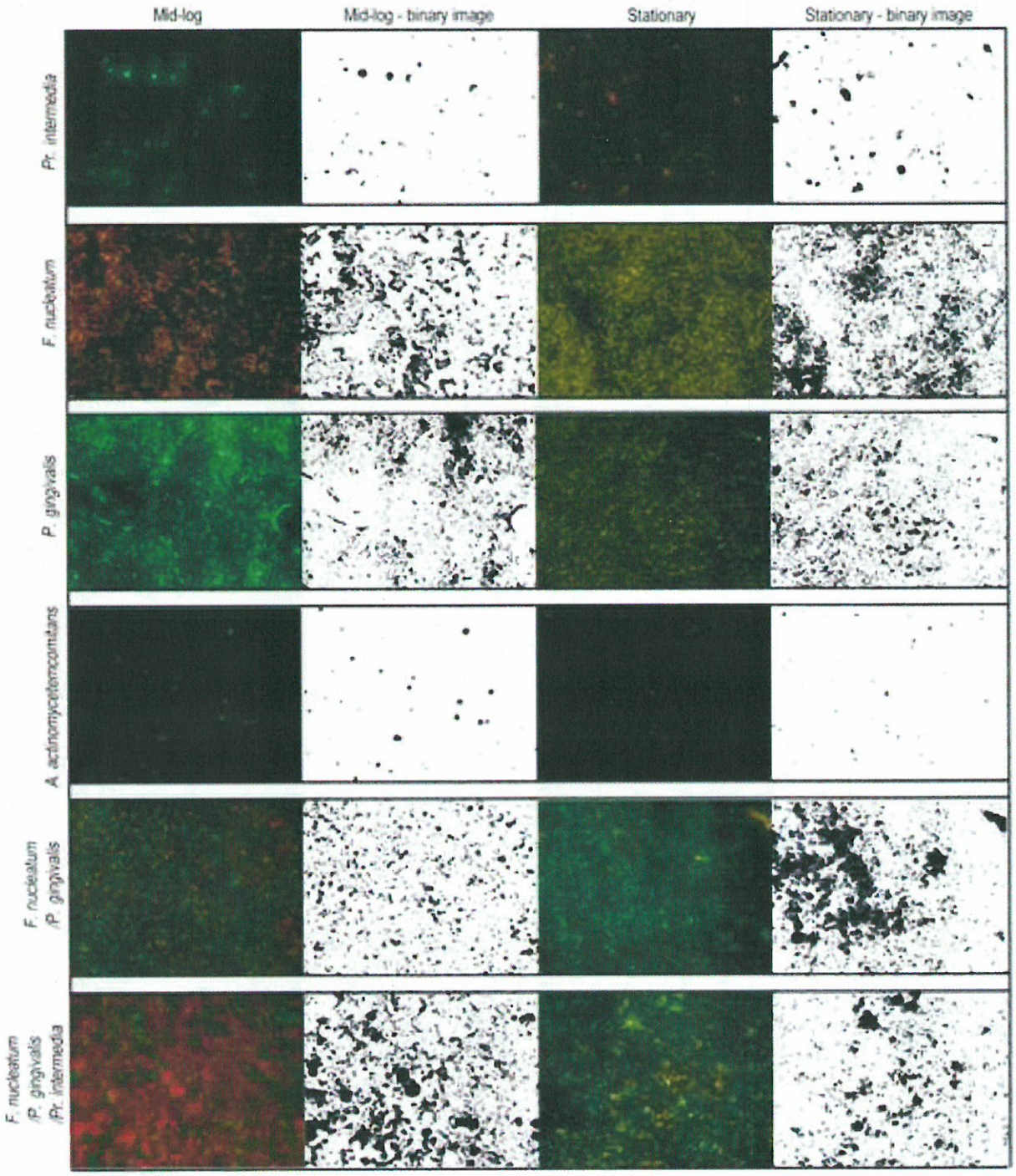


Figure 4

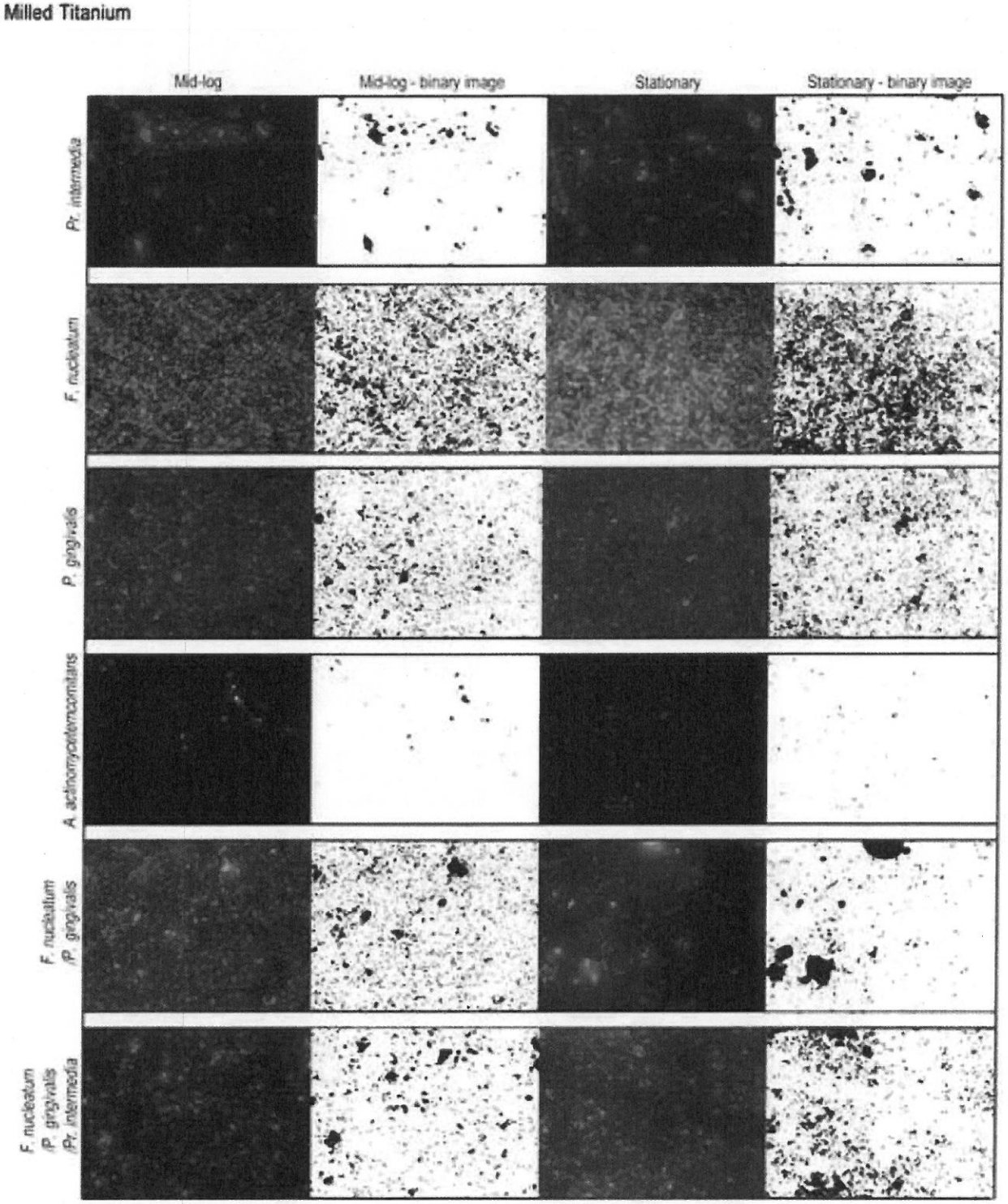
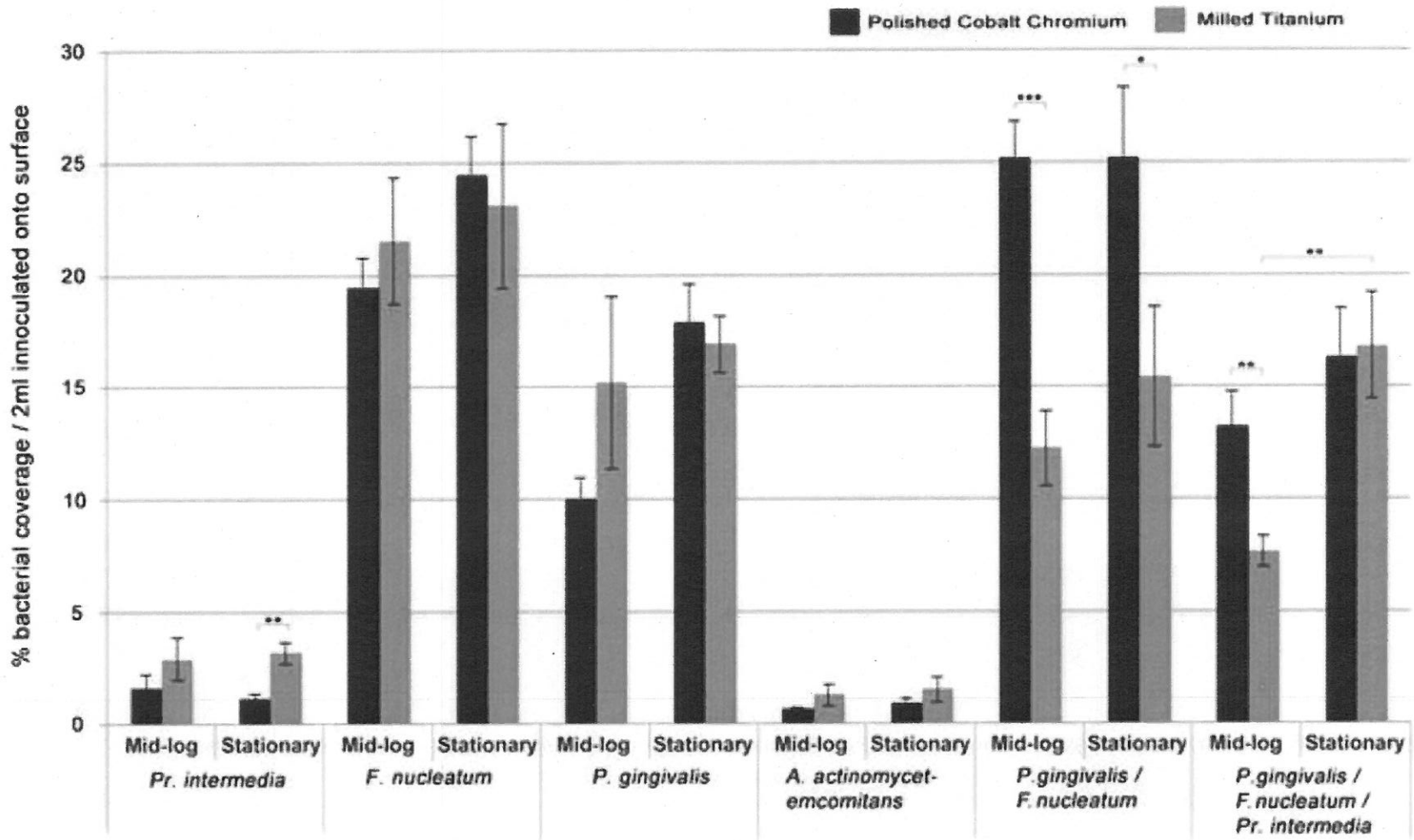

Milled Titanium
Mid-log
Mid-log - binary image
Stationary
Stationary - binary image
Pr. intermedia
F. nucleatum
P. gingivalis
A. actinomycetemcomitans
F. nucleatum /P. gingivalis
F. nucleatum /P. gingivalis /Pr. intermedia

Figure 5


Polished Cobalt Chromium
Milled Titanium
% bacterial coverage / 2ml innoculated onto surface
30
25
20
15
10
5
0
Mid-log
Stationary
Pr. intermedia
F. nucleatum
P. gingivalis
A. actinomycet-emcomitans
P.gingivalis / F.nucleatum
P.gingivalis / F.nucleatum / Pr. intermedia

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