An in vivo comparison of bacterial micro leakage in two dental implant systems: identification of a pathogenic reservoir in dental implants

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Keywords: Micro leakage, dental implants, bacteria, in vivo

Running head: Micro leakage in two dental implant systems

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Abstract

Objectives. The aim of this study was to compare bacterial leakage in two implant systems, one screw root form (SRF) with an external hexagon connection and one plateau root form (PRF) with a Morse taper internal connection. ***Materials and methods.*** Thirty two implants; 12 SRF and 20 PRF were sampled in fifteen patients. All implants had been in function for at least six months prior to sampling. The implant restoration was removed and 10µl of sterile saline was introduced into the implant well via a sterile glass syringe. The saline was drawn back up and transferred to the laboratory for microbiological analysis. The number of aerobic and anaerobic colony forming units per ml was determined and the dominant microorganism in each sample was identified by 16s rRNA gene amplicon sequencing. ***Results.*** There was a significant difference between bleeding on probing around the SRF implants (3%) and the PRF implants (28%) ($p=0.0496$). Bacterial microleakage was identified at 11 SRF and 19 PRF implants. The numbers of anaerobic bacteria recovered from PRF implants was significantly higher than that from SRF implants ($p=0.0002$). Streptococcus species and Enterococcus faecalis were found to dominate. ***Conclusions.*** This *in vivo* study demonstrated bacterial leakage in both types of implant systems, irrespective of the type of connection. Significantly greater anaerobic counts were found in the Morse taper internal connection implants.

Introduction

Dental implant success rates exceed 95%. Failures are attributed to mechanical or biological complications and are classified as early or late. One of the most common complications is peri-implantitis which is bacterial induced peri-implant bone destruction. Peri-implant mucositis presents as inflammation, with erythema, swelling and bleeding on probing around a fixture [1]. Prevalence of peri-implant mucositis has been reported in 80% of subjects and 50% of implants [2, 3]. Untreated peri-implant mucositis can progress to peri-implantitis and lead to failure of a dental implant. Peri-implantitis has been reported in 16-47% of subjects and 6-36% of implants, depending on the diagnostic criteria used [2, 4]. For accurate diagnosis, bleeding on probing, suppuration and increased probing depths are seen in conjunction with radiographic bone loss [1].

Bacteria have been identified on implant surfaces immediately following surgery [5]. Similar bacteria composition is found at healthy implants and adjacent natural teeth [6]. Analysis of bacteria at failing implants has found similar bacterial species to those identified at healthy sites, but with greater quantities of Gram negative anaerobic bacteria [7, 8].

The type of connection between an implant and an abutment can play an important role to the amount of bacterial leakage and the subsequent peri-implant inflammation and this has been demonstrated in vitro [9]. Histomorphometric analysis of biopsies of the tissues adjacent to the interface between the implant and abutment has revealed a marked inflammatory infiltrate and this was observed regardless of the degree of plaque accumulation [10]. Furthermore, a comparison of one piece and two piece implants revealed inflammation coronal to the interface in two piece implants that was not seen for one piece implants [11].

The microgap between the implant and abutment connection varies between implant systems and has been reported to be 20-50μm [12] although a subsequent study found the microgap to be in the region of 5μm [13]. Recently, a study reported an average gap distance of 1.7μm for a certain type of implant abutment connection. In this study though, the analysis was restricted only to the circumferential portion of the abutment-implant interface [14].

Most of the early implant designs featured an external hexagon connection which has been associated with peri-implant bone loss [15]. Bacteria have been identified on the apical aspect of abutment screws and internal surface of external connection Brånemark fixtures in vivo [16]. When Brånemark assemblies were immersed in broth containing bacteria, bacteria

microleakage was also observed in vitro [17]. In another study, a Morse taper internal connection implant was found to prevent bacterial microleakage in vitro for up to 72 hours [18]. Further in vitro studies have compared Morse taper implants to others with internal or external connections. A greater number of tri-channel internal connection implants were penetrated by bacteria [19] whilst no significant difference was found between Morse taper and an internal hexagon connection [20]. Morse taper connection implants showed significantly lower levels of microleakage than external connection implants in a study by Verdugo et al. (2013). Overall, most of the studies suggested that the amount of micro-leakage depends on the quality of the fit between the components, torque, and the forces applied during function [21, 22]

The purpose of this in vivo study was to compare bacterial microleakage in two implant systems that have been in function for at least six months. The aim was to assess if there was any difference in bacterial microleakage when comparing an implant system with an external hexagon connection (with the crown seated and the square Gold-Tite® torqued to 35 Ncm) to one with a Morse taper internal connection (integrated abutment crowns tapped into the implant).

Materials and methods

Ethical approval for the investigation was obtained from Trinity College Faculties of Health Science Ethics Committee in September 2010. Participants were recruited from the Dublin Dental School and Hospital. They had previously received one or more dental implants, each restored with a single crown, at least six months prior to the date of microbiological sampling. Participants with Plateau Root Form implants (Bicon® Boston, MA, USA) had been included in previous studies assessing patient satisfaction at the same institution (Figure 1). The Bicon® implant system uses a purely Morse tapered 1.5o implant abutment connection which relies on friction for retention. From the Participant with Screw Root Form (SRF) implants only implants with a screw retained restoration were included (Figure 2). All SRF implants had an external hexagonal connection (Biomet 3i™, Palm beach Gardens, FL, USA). The following participant exclusion criteria were applied; clinical evidence of current periodontitis or peri-implantitis [23], participant unable to attend for microbiological

sampling or unable to provide written consent and allergy to chlorhexidine. Patients were invited to attend an initial review appointment. Clinical examination was completed for all patients by a single operator (JM). Periodontal examination was carried out using a manual periodontal probe. Full mouth plaque scores (O'Leary) and presence/absence of bleeding on probing at all teeth and implants were recorded. Appropriate radiographs were taken if none had been taken within the previous twelve months.

Microbiological sampling: The implant to be assessed was isolated with sterile cotton wool rolls and suction. The restoration was removed according to implant brand. The Bicon® integrated abutment-crown was removed with forceps. The Biomet 3i™ crown was removed following removal of the restoration in the access cavity and unscrewing of the gold screw. All samples were taken immediately following removal of the abutment/crown. A 25µl sterile glass syringe (Hamilton Gastight syringe) was used to introduce 10µl of sterile saline into the well of the implant (as determined prior to the commencement of the study, the internal volume of the implants was able to accommodate saline volume in excess of 10µl). A sterile 22 gauge removable needle (51mm length) was used and discarded after each sample (Figure 3). The saline was immediately drawn back up and transferred into a sterile Eppendorf tube containing 250µl of sterile Brain Heart Infusion Broth (BHI). The implant was irrigated with copious chlorhexidine solution and dried. Any calculus present on the implant neck or restoration was removed. The abutment/crown was replaced according to manufacturers' instructions. The Bicon® integrated abutment crowns were tapped back into place and the occlusion was checked. The Biomet 3i™ crown was re-seated, the square gold screw (Gold-Tite®) replaced and manually torqued to 35 Ncm. The access hole was sealed with polytetrafluoroethylene tape and composite resin and the occlusion was checked.

Microbiological analysis: Samples were cultured on blood agar (BA) which consisted of Tryptic Soy Agar supplemented with 5 g/l yeast extract, 0.50 g/l L-cysteine hydrochloride, 5.0 µg/ml hemin and 1.0 µg/ml menadione and 5% horse blood (Oxoid). A 50 µl volume of neat sample and a 50 µl volume of sample diluted 1:10 in sterile BHI was plated in duplicate and incubated at 37˚C under aerobic conditions or at 37˚C in BBL gas jars under anaerobic conditions generated by AnaeroGen gas packs (Oxoid Limited, Wade Road, Basingstoke, Hampshire, England) for up to 5 days. Following the incubation period, the plates were removed and assessed for growth of bacterial colonies. A photographic record was made of

each plate using the colour camera on a colony counter (Flash&Go™) and the images were stored electronically for a manual count of colonies. (Figure 4). Bacterial densities were calculated and expressed as colony forming units per 10 µl sample (CFUs). The dominant colony type from each sample (i.e. that representing at least 60% of the colony morphologies present) was purified by subculture on BA until pure cultures were obtained. In order to identify the dominant colony type to the species level, we utilised to most accurate method currently available for this purpose; i.e. DNA sequence analysis of the 16s rRNA gene. Genomic DNA was prepared by transferring a single colony of each isolate to 2 ml Tryptic Soy Broth and incubation at 37°C to yield a saturated culture. A 1 ml aliquot was pelleted by centrifugation at 10,000 x g and DNA was prepared. The concentration of DNA was determined via Nanodrop software.

Amplification of the 16s rRNA gene was performed with the primers 27F (5'-AGAGTTTGATCC TGGCTGAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Weisburg et al.) Amplification was carried out with Go Taq polymerase (Promega Corp.) using standard cycling conditions and 10 ng of template DNA using a G-Storm™ thermal cycler. The presence of a single amplimer was confirmed by by electrophoresis in a 1% agarose gel and PCR samples were subsequently processed with the GeneElute PCR clean up kit (Sigma). DNA sequence analysis was carried out by Source BioScience using the same primer pair used for amplification. Presumptive identification of the bacteria was carried out by BLASTn search of the bacterial 16s rRNA data base at Human Oral Microbiome Database (HOMD, www.homd.org). Organisms were identified to the species level were a sequence match ≥98% could be identified in the database.

Statistical analysis: Fishers Exact test was used to compare the presence/absence of bleeding on probing at Biomet 3i™ and Bicon® implants. The aerobic/anaerobic counts were log transformed and a truncated regression model was used.

Results

Fifteen patients with a total of 32 implants consented for microbiological samples. The participants ranged in age from 22 to 82 years, reflecting the diversity of patients receiving dental implants in the institution (Table 1).

In total, 12 SRF and 20 PRF implants were sampled. A single patient (P8) had one implant of each type. All PRF implants were located in the premolar or first/second molar regions in accordance with a previous study protocol. All implants had been in function for at least six months prior to sampling (range 7-56months). Plaque scores, bleeding scores and full mouth mean probing depths were recorded for each patient and were no significant differences. During the study, it was observed that plaque and calculus were commonly found on the composite surface of the Bicon® integrated abutment-crowns and this might explain why there was more bleeding on probing around PRF than SRF implants (Table 2,3).

Bacterial microleakage, indicated by the recovery of bacteria from within the implant chamber following aerobic or anaerobic culture, was detected in 11 of 12 SRF implants sampled and 19 of 20 PRF implants sampled. The total number of CFUs recovered from each sample was calculated and the levels of bacterial contamination compared (Table 4). Only 2 of the 11 SRF (Biomet 3i™) implants sampled exhibited CFU counts greater than 250 whereas 17 of the 20 PRF (Bicon®) implants exhibited counts >250 CFU. Significantly higher counts of bacteria could be detected in the anaerobically incubated samples from PRF (Bicon®) implants compared to SRF (Biomet 3i™) implants ($p=0.0002$).

The dominant organism(s) from each sample was subcultured for identification by sequence analysis of the 16s rRNA gene. In total, the identities of 14 colonies from SRF and 20 colonies from PRF implants were determined. *Enterococcus faecalis* was the most frequently characterised microorganism in SRF (5 samples) and PRF (9 samples) type implants. The second most dominant organisms were *Streptococcus milleri* group (SMG) organisms, a species complex that includes *S. intermedius, S. sanguinus* and *S. constellatus* (Figure 5).

Discussion

This investigation demonstrated that regardless of the type of implant-abutment connection, bacterial micro leakage is a significant problem in dental implants. When bacterial plate counts recovered from SRF and PRF implants were compared, significantly greater counts were found in PRF implants. We utilised 16s rDNA sequencing to obtain an accurate identification of the dominant bacterial species recovered from each sample and found that

Streptococcus milleri group (SMG) organisms and *Enterococcus faecalis* were the dominant species present regardless of implant type. The dominance of these organisms in our cultures was unexpected as these species a have a relatively low abundance in the normal oral and subgingival microbiotas. This finding gives us confidence that our samples were not contaminated with saliva or GCF during sampling, as to have done so would have introduced large numbers of more common streptococci (*S. oralis, S. salivarius*) or common subgingival colonisers (e.g. fusobacteria, *Prevotella spp*.). It also suggests that the internal environment of the implant is highly selective for growth of a very specific microflora. The abundance of *E. faecalis* is perhaps not surprising due to the association of this species with endodontic infections. The anaerobic, nutrient poor environment of the pulp chamber following endodontic treatment may represent a similar environment to that found within the dental implant and this may be an environment where *E. faecalis* has the ability to thrive relative to other oral microorganisms. The samples were incubated aerobically and anaerobically using the AnaeroGen system. In general, the anaerobic incubations yielded higher numbers of CFUs relative to the aerobic cultures. This was an unusual finding given that the dominant organisms on the anerobic culture plates were in fact facultative organisms that should, in theory, grow well in aerobic culture. However, it must be noted that these organisms had been growing for many generations in the the anaerobic site of the implant chamber and it is likely that these organisms had adapted their metabolism for anaerobic growth. It is likely that the rapid switch to an aerobic envoroment, which would require induction of responses to toxic oxygen species such as superoxide dismutase, was a stressful event which may have lead to poor growth and lower recovery of CFUs relative to the anaerobic culture plates. Interestingly, we did not recover any true anaerobes in these samples. This is likely due to the restrictive nutrient environment of the implant chamber which would not support the growth of fastidious anaerobes such as *Porphyromonas spp.* and *Tannerella spp.* The culture system we use is routinely used for culture of species such as *P. gingivalis* so it is unlikely that out culture method prevented recovery of these species.

The results of the present study indicated that dental implants currently in use are a reservoir of pathogenic microorganisms with the potential to infect the implant sulcus or initiate other infections elsewhere. A variety of streptococcal species were detected in both implant systems. One third of Biomet 3i™ implants and 40% of Bicon® implants yielded streptococci as the most dominant bacteria. In the current study, the majority of streptococci identified were *Streptococcus milleri* group (*S. intermedius, S. sanguinus, S. constellatus*) or

S. mitis. The MGS can be aggressive pathogens in the head and neck region and a frequent cause of abscess [24]. Recent studies of the microbial communities associated with peri-implant disease have identified increased levels of non-mutans streptococci compared to healthy sites [25]. Our findings suggest that the implant chamber may act as a potential reservoir of non-mutans streptococci which may infect the implant sulcus if not routinely decontaminated. Additionally, *S. constellatus* has been identified at sites which have failed to respond to periodontal treatment [26]. Recently, *Streptococcus intermedius* has been associated with acute coronary syndrome [27]. However, the most frequently encountered organism in both implant types was *E. faecalis*, which was present in one third of Biomet 3i implants and 45% of Bicon implants. Enterococci are Gram positive facultative organisms which have been associated with endodontic lesions, particularly those with recurrent infection after treatment. They are capable of surviving in inhospitable environments and can utilise a variety of energy sources. Enterococci are normally found at relatively low levels in the normal flora of the mouth. Sedgley et al. detected *E. faecalis* in oral rinses from patients undergoing endodontic treatment more frequently than in those with no history of endodontic treatment [28]. *E. faecalis* has been identified in subgingival plaque samples taken from patients with chronic periodontitis which has failed to respond to treatment. Rams et al. found 5% of these patients harboured the bacteria which proved resistant to penicillin G, tetracycline, clindamycin and metronidazole [29]. *E. faecalis* has also been isolated from bone collected during bone augmentation and implant therapy [30]. Perhaps the most unexpected finding was the recovery of *S. pneumonia* in conjunction with *E. faecalis* in a PRF implant. *S. pneumonia* is a significant agent of respiratory disease and meningitis, and highlights the potential risk of serious infection associated with colonisation of the internal cavity by pathogenic organisms.

This study identified bacterial growth in both implant systems and supports the findings of Persson et al. who assessed Brånemark implants in 10 partially edentulous patients, in function for at least one year. They found that samples from the internal aspect of implants yielded substantial bacterial growth (>100CFU/0.1ml) with several species dominating each sample, predominately facultative streptococci [16]. Similar findings were reported by Keller et al. who detected Gram positive facultative bacteria in samples taken from the peri-implant sulci and the internal aspect of the prostheses of ITI implants [31]. Cosyn et al. found *Fusobacterium*, *Campylobacter* and streptococci colonizing the peri-implant sulcus and intra-coronal compartment of Brånemark implants which were in function for an average of 9

years. It was noted that intra-coronal quantities of all bacteria found were lower than the bacterial quantities in the sulcus. In addition, similar bacteria species were detected on the abutment screw, though again at lower quantities than in other regions [32]. Unlike these studies, our current study shows that *in vivo*, different implant types may harbour different levels of contamination. This may be related to the size of the micro-gap at the implant-abutment interface which not only regulates the transfer of microorganisms between the implant chamber and the environment, but also the level of nutrient exchange and the oxygen levels within the chamber. Several in vitro studies have attempted to compare bacterial microleakage in different dental implants. Duarte et al. compared inward movement of *E. faecalis* in five different implant systems, four of which featured external connections whilst the fifth had an internal connection. All five systems displayed bacterial leakage during the course of the investigation, regardless of the type of connection [33]. Jansen et al. compared outward leakage of *Escherichia coli* in nine different implant systems which presented with thirteen different implant-abutment combinations. All nine implant systems displayed bacteria leakage during the two week study period regardless of internal or external connection. Scanning electron microscope analysis of the microgap revealed it was less than 5µm for all systems used [13]. An in vitro comparison between two types of Morse taper connection implants by Aloise et al. found there was no difference in bacterial leakage between a tapped in connection (Bicon) and a screw connection (Ankylos). The leakage of *Streptococcus sanguinis* from within the implant assemblies was assessed and 20% of implants of both types of Morse taper connection displayed bacterial leakage during a 48 hour period. However, unlike these *in vitro* studies our *in vivo* study sugggests that connection type influences bacterial microleakage [34]. The study herein assessed implants which had been in function *in vivo* for at least 6 months prior to sampling. This may explain why bacterial leakage was observed more frequently than in some in vitro studies, which tend to be of short duration and use implant assemblies which are not subjected to loading. As a result the findings of *in vitro* studies may not accurately represent what occurs in functioning implants. In an attempt to overcome this limitation, Steinebrunner et al. utilised a chewing simulator to assess leakage of *Escherichia coli* in a variety of implant systems. All systems investigated displayed bacterial leakage [35].

The participants in this investigation were considered periodontally healthy, with bleeding index 3%, plaque index 29% and full mouth mean probing depth of 2.4mm. However, an incidental finding of this investigation was the observation of an inflamed collar of soft tissue

at Bicon implants, which was not seen at Biomet 3i™ implants. One third of all implants sampled had bleeding on probing at one or more sites. When the type of implant was considered, less than 10% of SRF implants demonstrated bleeding, compared to nearly 50% of PRF implants. This corresponds with the clinical observation of inflamed tissue. Also noted clinically was the presence of plaque deposits on the integrated abutment crown of Bicon® implants. It has previously been demonstrated that plaque accumulation at implants causes visible inflammation which is clinically demonstrated by bleeding on probing [36, 37]. One notable difference between the two types of implant systems studied was the material used to fabricate the crown. In the case of Bicon® integrated abutment crowns, a composite material was used. Biomet 3i™ crowns were composed of porcelain. Additionally the shape of the crowns differed, with Bicon® crowns being more bulbous, which may favour plaque accumulation in the absence of strict oral hygiene measures. Additionally, the Bicon® implants had been in function on average longer than the Biomet 3i™ implants.

Overall, the data presented here supports the idea that decontamination of the internal chamber during routine peri-implantitis treatment should be considered. Several authors have assessed the efficacy of chlorhexidine in reducing bacterial levels within the implant [38, 39]. Chlorhexidine has been applied as 1% gel to the internal cavity of implants and significantly reduced the bacterial load over a six month period [38]. Additional research has demonstrated a reduction in bacterial load on titanium discs in vivo when hydrogen peroxide, sodium hypochloride, 0.2% chlorhexidine or Listerine was applied [40].

Conclusions

Bacterial leakage was seen in both SRF and PRF implant systems and one could conclude that the Morse taper internal connection is incapable of preventing bacterial leakage *in vivo.* Ingress of bacteria was significantly greater in PRF implants. The organisms recovered have been associated with peri-implant infection and as discussed, this may have implications when providing treatment for peri-implantitis. We conclude that internal cleaning of the implant could be recommended as part of routine treatment for peri-implantitis.

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Table 1: Participant characteristics

	Biomet 3i™ N=6	Bicon® N=10	All N=15
Mean age (years) ± SD	59.0 (± 21.1)	48.9 (± 9.2)	52.4 (± 15.5)
Gender			12F:3M
Smoking status			
Never	3	6	8
Former	2	2	4
Current	3	0	3

Table 2: Clinical characteristics of patients

	Biomet 3i™ N=6*	Bicon® N=10*	All N=15
Bleeding score	2.17 ± 3.49	3.60 ± 2.22	3.13 ± 2.83
Plaque score	26.67 ± 19.88	30.00 ± 9.96	29.13 ± 14.33
Mean probing depth	2.15 ± 0.24	2.49 ± 0.34	2.37 ± 0.35

*=one patient had an implant of each type

Table 3: Presence/absence of bleeding on probing at implants

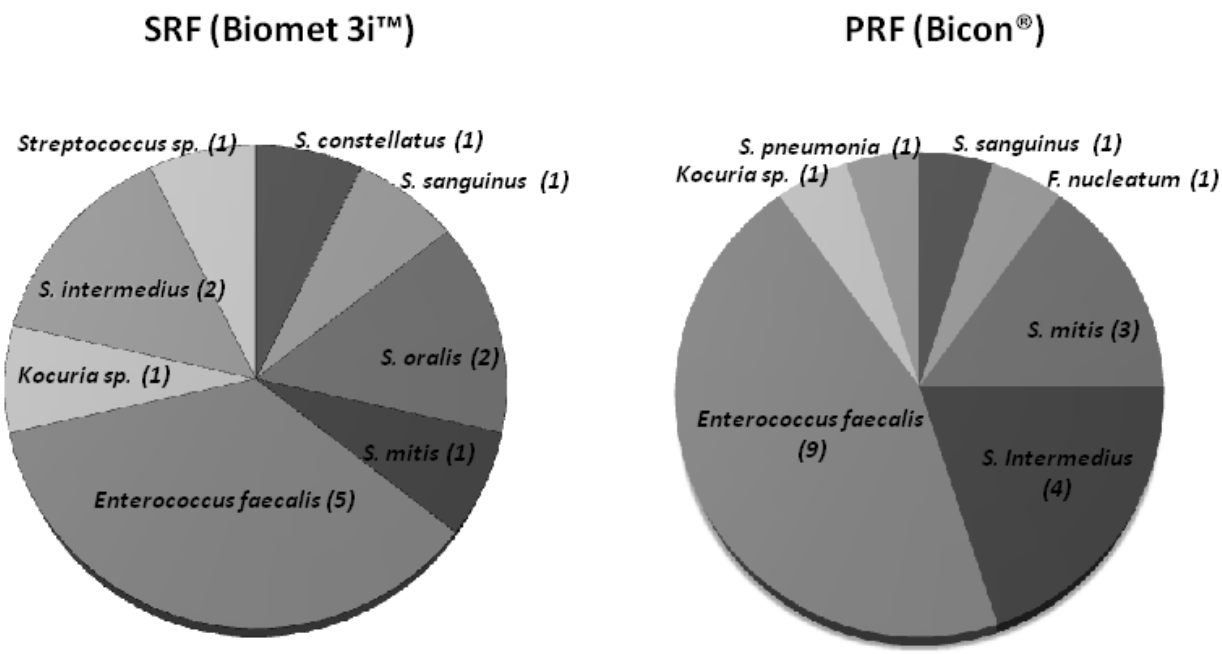
Bleeding on probing	Biomet 3i™	Bicon®	Total
+	1 (3%)	9 (28%)	10 (31%)
_	11 (34%)	11 (34%)	22 (69%)
Total	12	20	32

Fisher's Exact Test = 0.0496
95% confidence interval = 0.02973 to 1.346

Table 4: Table showing the breakdown of colony forming units (CFUs) in samples recovered from Biomet 3i™ and Bicon® implants.

	Implant Type			
	SRF (Biomet 3i™)		**PRF (Bicon)**	
CFUs	**Aerobic culture**	**Anaerobic culture**	**Aerobic culture**	**Anaerobic culture**
No Growth	3 (25%)	4 (33.3%)	2 (10%)	1 (5%)
1 to 250	7 (58.3%)	7 (58.3%)	12 (60%)	3 (15%)
251 to 2,000	0	0	0	8 (40%)
>2,000	2 (16.7%)	1 (8.4%)	6 (30%)	8 (40%)

Figure 1. Distribution of dominant bacterial species identified by 16s rRNA sequencing in SRF (Biomet 3i™) and PRF (Bicon®) implants.



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