CLINICAL ORAL IMPLANTS RESEARCH

Cássio do Nascimento
Murillo Sucena Pita
Paulo Linares Calefi
Thalisson Saymo de Oliveira Silva
Juliane Bustamante Sá dos Santos
Vinícius Pedrazzi

Different sealing materials preventing the microbial leakage into the screw-retained implant restorations: an *in vitro* analysis by DNA checkerboard hybridization

Authors' affiliations:
Cássio do Nascimento, Murillo Sucena Pita, Paulo Linares Calefi, Thalisson Saymo de Oliveira Silva, Juliane Bustamante Sá dos Santos, Vinícius Pedrazzi, Department of Dental Materials and Prosthodontics, Faculty of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

Corresponding author:
Prof. Dr Cássio do Nascimento
Faculdade de Odontologia de Ribeirão Preto
Universidade de São Paulo
Av. do Café s/n CEP: 14040-904
Ribeirão Preto, SP, Brazil
Tel.: +55 16 3315 4095
Fax: +55 16 3315 0547
e-mail: cassionasc@forp.usp.br

Key words: bacteria, dental implants, DNA checkerboard, microleakage, prosthodontics

Abstract

Objectives: The aim of this controlled *in vitro* study was to identify and quantify up to 38 microbial species penetrating through the screw-retained implant prostheses with different sealing materials.

Material and methods: Sixty morse cone implants were restored with single-unit screw-retained prostheses. All the components were randomly divided into five groups (n = 12) according to the proposed materials: (1) polytetrafluoroethylene tape+composite resin; (2) polytetrafluoroethylene tape+gutta-percha; (3) polytetrafluoroethylene tape+light-polymerized provisional composite; (4) cotton pellet+gutta-percha; and (5) cotton pellet+light-polymerized provisional composite. Human saliva was used as contaminant media, and DNA checkerboard hybridization was used to identify and quantify microbial species.

Results: Microbial leakage was observed in all groups: *M. salivarium*, *S. pasteuri*, *P. nigrescens*, and *P. melaninogenica* were the species presenting the highest values of genome count, prevalence, and proportion within the groups. The total microbial mean counts ($\times 10^5$, $\pm$SD) were as follows: Group 1 (2.81 $\pm$ 0.38), Group 2 (3.41 $\pm$ 0.38), Group 3 (6.02 $\pm$ 1.48), Group 4 (6.40 $\pm$ 1.42), and Group 5 (17.45 $\pm$ 1.67). Group 5 showed the higher microbial counts ($P < 0.001$).

Conclusions: Moderate to high counts of pathogenic/nonpathogenic species were detected in the inner parts of implants from all groups. The lowest values of microbial counts were recorded for polytetrafluoroethylene tape associated with composite resin or gutta-percha; cotton pellet associated with light-polymerized provisional composite presented the highest microbial counts.

The use of osseointegrated dental implants has been extensively reported in the literature as a safe and predictable treatment with high rates (>90%) of long-term success in single, partial, and complete restorations (Heydecke et al. 2012; Pjetursson et al. 2012; Kwon et al. 2014). Overall, this success should be attributed to a multifactorial condition, including the quality/quantity of bone, accurate treatment planning, proper oral hygiene, and the increase of physical, chemical, and mechanical properties related to the implants and components (Goiato et al. 2014; Pjetursson et al. 2014; Jimbo & Albrektsson 2015).

Despite the large diffusion of this treatment and the high survival rates in both partial and complete rehabilitations, several studies have reported over the years the presence of relevant microbial adhesion on the implants and components in health or disease (Augthun & Conrads 1997; Covani et al. 2006; do Nascimento et al. 2011; Yamane et al. 2013). During decades, most of the investigations including *in vitro* (do Nascimento et al. 2009, 2012) and clinical studies (Quirynen et al. 2005; Cosyn et al. 2011; Passos et al. 2013) have focused on reporting the bidirectional bacterial microleakage through the gaps resulting from the implant–abutment interface in both. In fact, the microbial colonization and consequent peri-implant tissues inflammation still constitute one of the major concerns since they are strongly associated with the early and late failure of dental implants (Palma-Carrió et al. 2011;

Date:
Accepted 30 December 2015

To cite this article:
do Nascimento C, Pita MS, Calefi PL, de Oliveira Silva TS, dos Santos JBS, Pedrazzi V. Different sealing materials preventing the microbial leakage into the screw-retained implant restorations: an *in vitro* analysis by DNA checkerboard hybridization.
Clin. Oral Impl. Res. **00**, 2016, 1–9
doi: 10.1111/clr.12790

Sakka et al. 2012). Additionally, in the screw-retained restorations, the hollow spaces resulting from the abutment screw access may also act as channels and reservoirs, harboring and favoring the colonization of microbial species present in the oral biofilm. In the past, studies proved the existence of this bacterial leakage along the access hole of the abutment (Quirynen & van Steenberghe 1993; Quirynen et al. 1994; Guindy et al. 1998). For this reason, the material used to seal the screw access channel and protect the abutment screw during the provisional time or in the definitive restoration is an important issue. Materials such as cotton, gutta-percha, vinyl polysiloxane, polytetrafluoroethylene tape, autopolymerizing acrylic resin, and composite resin have been used in protecting the abutment screw head and promoting the sealing of access hole (Adrian et al. 1991; Taylor et al. 2004; Weininger et al. 2008). In these studies, the authors have described the characteristics of each material and their impact in the mechanical properties of assemblies. However, there is limited information on the effect of these different sealing materials in preventing bacterial/fluids microleakage. Recently, Park et al. (2012) evaluated the levels of fluids leakage through the access holes of screw-retained implant prostheses sealed with cotton, silicone, vinyl polysiloxane, or gutta-percha. Vinyl polysiloxane and gutta-percha showed the lower levels of leakage. In a similar study, Cavalcanti et al. (2015) investigated the efficacy of gutta-percha and polytetrafluoroethylene tape in sealing the penetration of *Escherichia coli* along the abutment screw access hole; gutta-percha was shown significantly superior. Currently, there is no consensus or defined protocols for this objective. Thus, we aimed in this controlled *in vitro* study, by means of the sensitive checkerboard DNA–DNA hybridization, to identify and quantify up to 38 microbial species penetrating through the screw-retained implant prostheses with different sealing materials. The null hypothesis tested was that there is no significant difference between sealing materials in preventing the microbial leakage through the abutment screw access hole.

Material and methods

Preparation of the specimens

A total of 60 implants with a morse cone (MC) connection (3.8 mm Ø, 10 mm long, SIN® – Sistema de Implante Nacional, São Paulo, Brasil) were used in this investigation. Sixty conical abutments (2 mm in height, SIN) for screw-retained prosthetic restorations, engaging the appropriate antirotational interface geometry, were selected to be attached to the implants. All the components were target and randomly divided into five groups ($n = 12$), by means of computer-generated numbers, according to the proposed sealing materials and protocols: (1) polytetrafluoroethylene tape + composite resin; (2) polytetrafluoroethylene tape + gutta-percha; (3) polytetrafluoroethylene tape + light-polymerized provisional composite; (4) cotton pellet + gutta-percha; and (5) cotton pellet + light-polymerized provisional composite. The specifications of materials used in this study are displayed in Table 1.

Each experimental specimen was constituted by an implant restored with a single-unit fixed prosthesis. Sixty premachined cobalt-chromium alloy (Co-Cr) cylinders with plastic sleeves (SIN) were used to fabricate identical metallic single-molar crowns (8 mm in height, 10 mm mesiodistal length, 6 mm buccolingual length) with an occlusal screw access opening to abutment screw tightening. The prostheses were cast with the same amount of cobalt-chromium alloy, divested, and airborne-particle abraded with 100-μm aluminum oxide (55 N/cm^2). A layer of 0.5 mm of feldspathic porcelain (IPS Classic V Dentin, Ivoclar-Vivadent, Schaan, Liechtenstein) was applied on the occlusal aspect of the crowns to simulate a metalloceramic restoration surface. The final measures of the screw access hole to be filled in this investigation were 2.5 mm in diameter and 1.5 mm in height (from the upper part of the abutment screw to the occlusal aspect of the crown).

The implants were embedded in an autopolymerizing resin (VIPI, Pirassununga, SP, Brasil) with a custom-made acrylic ring form (20 mm in diameter and 15 mm in height). They were inserted using a parallelometer, allowing the same position and inclination for all the specimens. This procedure was carried out aiming to create a contamination chamber and to standardize the specimens' position during incubation in the contaminant media.

Microbiological assessment

In an effort to simulate the oral cavity conditions, human saliva was used as a contaminant media for specimens' incubation. Five milliliters of nonstimulated saliva were collected from 24 healthy individuals (mean age 33 years) and mixed into a single Falcon tube. Additionally, samples of supragingival biofilm from first superior and inferior molars of the participants were taken with curettes and added to the saliva to increase the final microbial concentration in the contaminant media. The participants enrolled in this study had no clinical signs of diseases in the oral mucosa. The gingival sulci had less than 3 mm deep, without clinical signs of gums inflammation. There were no caries or active white spot lesions in the teeth. Additional exclusion criteria were pregnancy, lactation, periodontal or antibiotic treatment in the earlier 3 months, current smokers, or any systemic disease which could influence the periodontal status. The study was approved by the local ethics committee, and all the experiments were undertaken with the understanding and written consent of each subject and according to the ethical principles.

All the embedded implants and their metallic crows were sterilized with hydrogen peroxide plasma during 60 min. Abutments were sterilized by manufacturers. Before implant–abutment attachment, contents of the internal parts of the sterilized implants were collected with sterile microbrushes to be used as negative control for contamination. Under aseptic conditions, the abutments were attached to the implants using a manual torque meter. The titanium abutment screws were tightened to 20 Ncm in all connections and single crowns at 10 Ncm, as instructed by the manufacturer. The occlusal screw accesses of the prostheses were sealed according to the protocols established during groups randomization: (1) Polytetrafluoroethylene Tape + Composite Resin, simulating definitive restoration; (2) Polytetrafluoroethylene Tape + gutta-percha;

Table 1. **Sealing materials used in this investigation**

Material	Specification	Manufacturer
PTFE tape	Polytetrafluoroethylene tape	Tigre S/A, São Paulo, SP, Brazil
Polar fix	Cotton pellet	Polar Fix, Ribeirão Pires, SP, Brazil
Filtek Z250	Composite resin	3M ESPE, Saint Paul, MN, USA
Gutta-percha sticks	Gutta–percha	Dentsply Maillefer, Ballaigues, Switzerland
Bioplic	Light-polymerized provisional composite	Biodinâmica LTDA, Ibiporã, PR, Brazil

(3) Polytetrafluoroethylene Tape + Light-Polymerized Provisional Composite; (4) Cotton Pellet + gutta-percha; and (5) Cotton Pellet + Light-Polymerized Provisional Composite, simulating temporary restoration. Polytetrafluoroethylene tape and cotton pellet were restricted to fulfill the hollow space of the abutment screw head protecting from composite resin, light-polymerized provisional composite, or gutta-percha, as in the clinical practice.

After treatments, the specimens were connected to rubber tubes through the acrylic resin basis. The tubes were sealed with cyanoacrylate adhesive and acted as a reservoir to the human saliva during incubation period. They are positioned so that the contaminant media was limited to the abutment screw access. The specimens were totally immersed in 1 ml of human saliva and incubated at 37°C in microaerophilic conditions during 7 days. A set of 12 samples of 150 μl of the human saliva was collected before and after the period of incubation and tested against the target probes to verify the presence of the target microorganisms proposed for evaluation. After 7 days of incubation, the specimens were removed from rubber tubes and rinsed with 0.9% of saline solution and 70% ethanol to remove external contamination. After drying and sealing removal, the specimens were opened under aseptic conditions, and the contents of the inner parts of the implants and screw threads were collected with sterile microbrushes. All the specimens were transferred to microtubes containing 150 μl of TE (10 mM Tris-HCl, 1 mM EDTA pH 7.6) followed by the addition of 150 μl of 0.5 M NaOH. The occurrence of microbial leakage through the screw access channel interface was assessed with the checkerboard DNA–DNA hybridization method proposed by Socransky et al. (1994) with a modification according to do Nascimento et al. (2010). Thirty-seven bacterial species including putative periodontal pathogens (*Porphyromonas gingivalis*, *Tannerella forsythia,* and *Treponema denticola*) and *Candida* spp., frequently found harboring the oral microbiota of healthy and diseased individuals, were investigated (Table 2). Briefly, after 5 min of boiling, microtubes containing samples were cooled and mixed with 800 μl of 5 M ammonium acetate. The contents of each tube were individually concentrated onto a nylon membrane (Hybond N+; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and baked for 2 h at 80°C. As standard references, defined amounts of genomic DNA corresponding to either 10^5 or 10^6 microbial cells of each species evaluated were also applied on the same membrane set. The membranes were prehybridized at 60°C for 2 h in the hybridization solution [NaCl 0.5 M; Blocking reagent 0.4% (w/v)]. After prehybridization, defined amounts of labeled whole genomic probes were applied on each individual lane into the membrane. Hybridization process was performed overnight at 60°C. After washing, the hybridization signals were detected by chemiluminescence using CDP-*Star* (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Signals were registered by exposing the membranes to ECL Hyperfilm-MP (GE Healthcare). The image obtained on Hyperfilm was digitized and analyzed with the TotalLab Quant software (TotalLab, Newcastle upon Tyne, England). The estimated number of microorganisms recovered from the internal part of the implants could be expressed in terms of genome counts.

Data analysis

Descriptive statistics was used to analyze the genome counts and frequency of positive signals of hybridization. The Kruskal–Wallis one-way analysis of variance by rank test for original-level data was used to compare genome counts, prevalence, and proportions of each target species, for independent measures. When applicable, post hoc analysis by means of Bonferroni's test was performed for multiple comparisons. Differences were considered significant when $P < 0.05$. The SPSS 17.0.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis.

Results

No positive signals of hybridization were registered for samples collected to be used as negative control. The human saliva collected before incubation showed higher microbial genome counts when compared with saliva collected after the incubation period ($P < 0.001$), but no differences were observed in relation to the prevalence of target species ($P > 0.05$). All of the target species were present in relevant counts in the saliva after the incubation period.

The presence of microbial leakage through the abutment screw access was observed in all groups evaluated after 7 days of human saliva incubation. The minimum, maximum, median, lower, and upper quartiles of microbial genome counts ($\times 10^5$ cells) of the 38 target species and their respective *P*-values are displayed in Table 3. Kruskal–Wallis test showed significant differences comparing medians of target species within the different materials and protocols ($P < 0.0001$ for most of the species and $P = 0.0297$ for *B. fragilis*). Only *C. gingivalis* showed no significant differences between treatments ($P = 0.0585$). Bonferroni's multiple comparisons post-tests showed most of the target species presenting significant differences comparing medians from different groups, except *B. fragilis*. *M. salivarium* recorded the most significant differences; medians from Groups 2 (4.72) and 5 (4.25) were higher than medians from Groups 1 (0) and 4 (0) ($P < 0.001$); *S. pasteuri* recovered from Group 5 (4.05) presented higher median when compared to Groups 1 and 4 (0; $P < 0.001$); *P. melaninogenica* from

Table 2. **Target microbial species and respective ATCC number**

Species	ATCC number	Species	ATCC number
Aggregatibacter actinomycetemcomitans	29523	*Pseudomonas putida*	12633
Bacteroides fragilis	25285	*Staphylococcus aureus*	25923
Capnocytophaga gingivalis	33624	*Streptococcus constellatus*	27823
Campylobacter rectus	33238	*Streptococcus gordonii*	10558
Escherichia coli	10798	*Streptococcus mitis*	49456
Eikenella corrodens	23834	*Solobacterium moreei*	CCUG39336
Enterococcus faecalis	51299	*Streptococcus mutans*	25175
Fusobacterium nucleatum	25586	*Streptococcus oralis*	35037
Fusobacterium periodonticum	33693	*Streptococcus parasanguinis*	15911
Klebsiella pneumoniae	700603	*Staphylococcus pasteuri*	51129
Lactobacilos casei	393	*Streptococcus salivarius*	25975
Mycloplasma salivarium	23064	*Streptococcus sanguinis*	10556
Neisseria mucosa	25996	*Streptococcus sobrinus*	27352
Pseudomonas aeruginosa	27853	*Treponema denticola*	35405
Peptostreptococcus anaerobius	27337	*Tanerella forsythia*	43037
Porphyromonas endodontalis	35406	*Veillonella parvula*	10790
Porphyromonas gingivalis	33277	*Candida albicans*	10231
Prevotella intermedia	25611	*Candida dubliniensis*	44508
Prevotella melaninogenica	25845	*Candida glabrata*	66032
Parvimonas micra	33270	*Candida krusei*	2159
Prevotella nigrescens	25261	*Candida tropicalis*	13803

Table 3. **Minimum, maximum, median, lower, and upper quartiles of microbial genome counts (×10^5 cells) and respective *P*-values assessed by checkerboard DNA–DNA hybridization in tested groups**

	Group 1					Group 2					Group 3					Group 4					Group 5					
	PTFE + Composite resin					PTFE + Gutta-Percha					PTFE + Provisional composite					Cotton + Gutta-Percha					Cotton + Provisional composite					
	Minimum	Maximum	Lower quartile	Median	Upper quartile	Minimum	Maximum	Lower quartile	Median	Upper quartile	Minimum	Maximum	Lower quartile	Median	Upper quartile	Minimum	Maximum	Lower quartile	Median	Upper quartile	Minimum	Maximum	Lower quartile	Median	Upper quartile	*P*-value
Candida spp.*	0	4.50	0	0	3.35	0	5.10	0	0	0	0	11.58	0	0	0	0	3.50	0	0	0	0	4.09	0	0	3.15	<0.0001
*V. parvula**	0	0.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.63	0	0	3.00	<0.0001
*T. denticola**	0	5.40	3.39	4.11	4.49	0	0	0	0	0	0	0	0	0	0	0	4.55	0	0	0	0	3.60	0	0	2.08	<0.0001
*T. forsythia**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.64	0	0	0	0	3.90	0	0	3.07	<0.0001
*S. sobrinus**	0	3.50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.43	0	0	0	0	3.91	0	2.62	3.22	<0.0001
*S. sanguinis**	0	3.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.78	0	1.53	3.57	0	3.77	0	0	3.12	<0.0001
*S. salivarius**	0	2.99	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.82	0	0	0	0	3.30	0	0	2.82	<0.0001
*S. pasteuri**	0	2.93	0	0	0	0	6.21	0	4.89	5.26	0	5.51	2.95	3.94	4.89	0	0	0	0	0	0	5.16	3.61	4.05	4.56	<0.0001
*S. parasanguinis**	0	3.12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.42	0	0	0	0	3.81	0	2.83	3.50	<0.0001
*S. oralis**	0	2.99	0	0	0	0	5.15	0	0	0	0	0	0	0	0	0	2.78	0	0	0	0	4.57	0	3.27	3.54	<0.0001
*S. mutans**	0	0	0	0	0	0	4.53	0	0	0	0	0	0	0	0	0	3.86	0	0	0	0	4.41	2.97	3.26	3.62	<0.0001
*S. moorei**	0	0	0	0	0	0	4.26	0	0	0	0	0	0	0	0	0	4.95	0	1.18	3.25	0	3.77	0	3.12	3.56	<0.0001
*S. mitis**	0	0	0	0	0	0	4.08	0	0	0	0	0	0	0	0	0	5.50	0	0	0	0	3.60	0.68	3.11	3.47	<0.0001
*S. gordonii**	0	3.82	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.83	2.66	3.33	3.81	0	4.78	0	2.94	3.21	<0.0001
*S. constellatus**	0	3.14	0	0	0	0	3.67	0	0	0	0	0	0	0	0	0	3.06	0	0	0	0	4.04	0	2.96	3.35	<0.0001
*S. aureus**	0	4.50	0	1.43	3.39	0	4.28	0	0	0	0	0	0	0	0	0	3.34	0	2.57	3.09	0	4.96	0.78	3.48	3.74	<0.0001
*P. putida**	0	0	0	0	0	0	4.04	0	0	0	0	0	0	0	0	0	3.63	0	0	0	0	5.63	0	2.92	3.20	<0.0001
*P. nigrescens**	0	14.75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.49	3.76	4.33	4.74	0	3.83	0	2.56	2.91	<0.0001
*P. micra**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.98	0	0	2.61	<0.0001
*P. melaninogenica**	0	6.64	0	5.32	5.65	0	4.57	0	0	0	0	6.57	0	0	5.15	0	4.01	0	0	0	0	3.45	0	2.49	2.78	<0.0001
*P. intermedia**	0	3.55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.72	0	3.50	4.10	0	3.12	0	0	2.75	<0.0001
*P. gingivalis**	0	3.29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.98	0	0	3.24	0	3.54	0	0	2.92	<0.0001
*P. endodontalis**	0	3.28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.49	0	0	0	0	3.67	0	2.87	3.20	<0.0001
*P. anaerobios**	0	3.69	0	0	3.61	0	0	0	0	0	0	0	0	0	0	0	3.63	0	0	0	0	3.63	0	2.66	3.18	<0.0001
*P. aeruginosa**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.53	0	0	0	0	3.90	0	3.01	3.40	<0.0001
*N. mucosa**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.54	0	3.04	3.53	<0.0001
*M. salivarium**	0	3.27	0	0	0	0	6.62	3.96	4.72	5.50	0	5.47	0	0	0	0	0	0	0	0	0	6.97	3.54	4.25	4.57	<0.0001
*L. casei**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.71	0	2.88	3.09	<0.0001
*K. Pneumoniae**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.15	0	0	3.56	0	3.52	0	1.33	2.99	<0.0001
*F. periodonticum**	0	3.66	0	0	0	0	4.10	0	0	0	0	0	0	0	0	0	4.52	0	3.29	3.82	0	3.37	0	0	2.69	<0.0001
*F. nucleatum**	0	4.50	0	0	3.93	0	0	0	0	0	0	0	0	0	0	0	4.79	0	0	0	0	3.51	0	2.66	3.07	<0.0001
*E. faecalis**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.21	0	0	2.89	<0.0001
*E. corrodens**	0	3.76	0	0	0	0	4.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.55	0	2.68	3.07	<0.0001
*E. coli**	0	3.43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.63	0	2.78	2.94	<0.0001
*C. rectus**	0	3.72	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.52	0	1.27	2.96	<0.0001

Table 3. (continued)

	Group 1					Group 2					Group 3					Group 4					Group 5					
	PTFE + Composite resin					PTFE + Gutta-Percha					PTFE + Provisional composite					Cotton + Gutta-Percha					Cotton + Provisional composite					
	Minimum	Maximum	Lower quartile	Median	Upper quartile	Minimum	Maximum	Lower quartile	Median	Upper quartile	Minimum	Maximum	Lower quartile	Median	Upper quartile	Minimum	Maximum	Lower quartile	Median	Upper quartile	Minimum	Maximum	Lower quartile	Median	Upper quartile	*P*-value
C. gingivalis	0	4.12	0	0	2.25	0	4.03	0	0	0	0	0	0	0	0	0	3.72	0	0	0	0	2.84	0	0	2.71	0.0585
B. fragilis	0	3.85	0	0	0	0	4.80	0	0	0	0	0	0	0	0	0	4.67	0	0	0	0	3.14	0	0	2.80	0.0297
*Aa**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.31	0	0	2.89	0	3.76	0	0	2.79	<0.0001

*Species with differences between tested groups detected by Bonferroni's Multiple Comparisons Post-tests ($P < 0.05$).
PTFE, Polytetrafluoroethylene; *Aa*, *Aggregatibacter actinomycetemcomitans*.

Group 1 (5.32) presented higher median than Group 4 (0; $P < 0.001$); and *P. nigrescens* from Group 4 (4.33) recorded higher median than Group 1 (0; $P < 0.001$). The highest mean values ($\times 10^5$ cells, ±SD) of genome counts were recorded for *M. salivarium* in samples from Group 2 (4.74 ± 0.87), followed by *P. nigrescens* (4.17 ± 1.06) from Group 4, *M. salivarium* from Group 5 (4.09 ± 0.1.25), and *P. melaninogenica* from Group 1 (3.91 ± 2.60).

The total microbial genome counts were also provided by assessing a pool of all the 38 microbial species in each group. Fig. 1 illustrates medians with interquartiles range of species found colonizing the tested groups. Significant differences were found between medians (Kruskal–Wallis test; $P < 0.0001$). Group 5 showed the higher microbial count compared to the other groups ($P < 0.001$; Bonferroni's multiple comparisons post-tests). Groups 3 and 4 presented higher counts when compared with Groups 1 and 2 ($P < 0.001$). The total mean counts (±SD) for each group were recorded as follows: Group 1 (2.81 ± 0.38), Group 2 (3.41 ± 0.38), Group 3 (6.02 ± 1.48), Group 4 (6.40 ± 1.42), and Group 5 (17.45 ± 1.67).

The mean counts ($\times 10^5$ cells), proportion (%), and prevalence (%) of each target species in the tested groups are displayed in Table 4. *P. nigrescens*, *S. mutans*, *S. pasteuri*, and *M. salivarium* showed significant differences between tested groups (Bonferroni's post-test; $P < 0.05$). Overall, the highest values of prevalence were recorded for species from Group 5. The highest prevalence was recorded for *M. salivarium* (100%) in Group 2, followed by *S. pasteuri* and *S. mutans* in Group 5 (95.83%). *M. salivarium* ($P < 0.001$), *S. pasteuri* ($P < 0.001$), and *P. melaninogenica* ($P < 0.05$) were shown presenting significant differences in the proportions when compared within the different groups.

Discussion

In terms of the microbial leakage associated with the abutment screw access channel, the literature is still rather scarce. Considering the relevance of this subject to the long-term success of implant-supported restorations, we investigated in this controlled *in vitro* study the microbial leakage of up to 38 different oral microbial species through the abutment screw access hole by means of a sensitive genetic material-based method of microbial detection and quantitation. The leakage of several bacterial species and *Candida* spp., commonly found in the oral microbiota was observed in all groups evaluated, irrespectively sealing materials proposed. Groups presented differences in relation to the total microbial genome counts after saliva incubation; prevalence and proportions of determined species were also divergent between groups. Thus, the null hypothesis tested in this investigation was rejected.

Overall, implant-retained restorations restored with polytetrafluoroethylene tape showed the lower counts of microorganisms recovered from the inner parts of the implants after saliva incubation ($P < 0.0001$). The lowest mean values of total microbial count ($\times 10^5$ cells, ±SD) were recorded when polytetrafluoroethylene tape was associated with composite resin (Group 1; 2.81 ± 1.23) or gutta-percha (Group 2; 3.41 ± 1.22), without differences between them. In contrast, groups restored with cotton pellets presented higher microbial counts; the highest mean

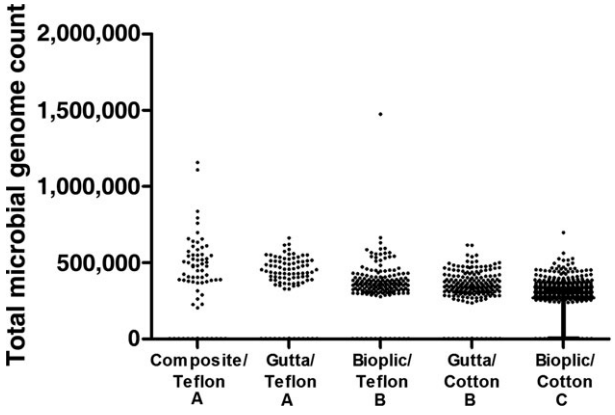

Fig. 1. Scatter plot of the total microbial genome count with median and interquartile range for tested groups (Different letters mean significant differences detected by Kruskal–Wallis test followed by Bonferroni's multiple comparisons post-tests; A < B < C $P < 0.001$).

values were recorded for Group 5 when cotton pellet was associated with the light-polymerized provisional composite (17.45 ± 1.67). The mean total genome count values of the studied materials were ordered as follows: Group 1 (Polytetrafluoroethylene Tape + Composite Resin) = Group 2 (Polytetrafluoroethylene Tape + gutta-percha) < Group 3 (Polytetrafluoroethylene Tape + Light-Polymerized Provisional Composite) = Group 4 (Cotton Pellet + gutta-percha) < Group 5 (Cotton Pellet + Light-Polymerized Provisional Composite).

The species found colonizing the internal parts of the implants included nonpathogenic and pathogenic microorganisms. Pathogenic species from oral "red complex" (*P. gingivalis*, *T. forsythia*, and *T. denticola*), which are closely related to the periodontal/peri-implantar diseases, were recovered in lower levels in all groups. *M. salivarium*, *S. pasteuri*, *P. nigrescens*, and *P. melaninogenica* were the species presenting the highest values of genome count, prevalence, and proportion within the groups. These findings are of clinical relevance as the increased colonization of *Prevotella* spp. in peri-implant sulci has been related to peri-implantitis (Zhuang et al. 2014; Albertini et al. 2015; Neilands et al. 2015). In addition, *Prevotella* spp. and other species found in moderate to high levels in our investigation (*S. constellatus*, *F. nucleatum*, *P. micra*, and *C. rectus*) constitute the "orange complex" and are considered precursors for the "red complex" colonization. Both complexes are associated with clinical parameters of peri-implant diseases (Socransky et al. 1998).

It has been reported that various materials have been used filling the screw access channels of the abutments of screw-retained implant-supported restorations (Moráguez & Belser 2010; Park et al. 2012; Cakan et al. 2014; Cavalcanti et al. 2015). However, few studies have focused on the ability of these materials in preventing or minimizing the microbial/fluids leakage through the abutment screw access hole. Park et al. (2012) investigated the occurrence of fluids leakage through this pathway testing different sealing materials, including cotton pellet and gutta-percha. They evaluated the penetration of basic fuchsin into the internal part of the implants after loading simulation. Corroborating to our results, irrespective of differences on the experimental design and methodologies, they found that microleakage associated with cotton pellet was significantly higher than gutta-percha. In a recent microbiological assessment, Cavalcanti et al. (2015) investigated the efficacy of gutta-percha and polytetrafluoroethylene tape in preventing the penetration of *E. coli* through the prosthetic abutments of hexagonal hex or morse cone implants. Similar to our results, they found that none of the proposed sealing materials could prevent the microbial leakage through the components; also, the authors concluded that gutta-percha was more effective than polytetrafluoroethylene tape minimizing the leakage. Both investigations found that type of sealing used influenced the fluids/microbial penetration as well as confirmed in the present study.

Different from these previous investigations, in the present study, we proposed to test combined materials in the filling of abutment screw access openings. Our experimental design was structured based on a recent literature report, in which Tarica et al. (2010) performed 68 surveys in a total of 62 US dental schools. Questionnaires consisting of eight common questions related to the implant-supported restorations were applied in restorative departments, advanced prosthodontics programs, and implant programs. Most of the schools (32%) responded that they use two associated materials to fill out the channel while 16% used only one material. According to this study, the five most commonly reported sealing materials were cotton pellets, composite resin, gutta-percha, rubber-based material, and light-polymerized provisional composite. 77% of schools responded that they fill the channel, either partially or entirely, with a cotton pellet protecting the abutment screw head; composite resin was the most frequent restorative material.

In our investigation, we judged not relevant to carry out a loading simulation considering that microorganisms penetrating through the interface between sealing materials and restorations in an unloaded condition reflect a more critical situation for the tested materials. The presence of external forces results in internal tensions that may cause failure of the materials. In this case, the number and size of gaps in the interface may be increased affecting the final rate of the microbial leakage. The specimens were constructed so that the contaminant media (human saliva) was limited to the abutment screw access. No contaminant media was in contact with the implant–abutment interface. Our data proved that sealing materials cannot prevent the microbial leakage from the external environment to the inner parts of the implants, even in the absence of loading. It is not surprising, once the average diameters of the smallest bacterial species found in the oral biofilm (i.e., *E. coli*) range from 0.7 to 1.1 μm. Studies have shown that bacterial species can grow, move, and penetrate very narrow constrictions with a size comparable to or even smaller than their diameter (Männik et al. 2009). Although the level of acceptable marginal misfit is still controversial in dentistry, values up to 120 μm are considered clinically acceptable for fixed prosthodontics (Kosyfaki et al. 2010). Additional analysis of marginal gaps between sealing materials and restorations by scanning electron microscopy (SEM) may help in elucidating these findings.

The use of human saliva as contaminant media in conjunction with checkerboard DNA–DNA hybridization methodology allowed the evaluation of a broad spectrum of microbial species colonizing specimens after incubation. This methodology allowed the simultaneous identification and quantitation of the entire 38 target species proposed in this investigation. It was very interesting because, as known, the oral biofilm consists of a complex microbiota composed of bacterial and fungal species enrolled in a matrix of proteins, extracellular products, and other salivary compounds. All of these characteristics in conjunction may influence the survival and way of leakage of microorganisms. In addition, this methodology consists in the identification of microorganisms by genetic material allowing both viable and nonviable cells to be detected.

A limitation of the methodology used for microbial identification is the minimal number of microbial cells to be detected (10^4 cells), meaning that counts in the range 1–1000 could have been present in the specimens but went undetected by the method. In this case, microorganisms should not really be present or be found in extremely reduced counts. Other possible rationale for the reduced counts or no detection is the occurrence of genetic material degradation as a result of the action of the proteolytic enzymes and endonucleases released by cell death or unfavorable environment (Katsoulis et al. 2005). Supplemental information from more sensitive DNA-based methods, such as real-time PCR, would benefit from adding important new information on the data provided by DNA checkerboard.

The findings obtained in this investigation may be of clinical relevance as we proved that a wide variety of oral microorganisms may penetrate into the implant

Table 4. **Mean genome count (×10^5 cells), proportion (%), and prevalence (%) of individual target species**

	Group 1			Group 2			Group 3			Group 4			Group 5		
	PTFE + Composite resin			PTFE + Gutta-Percha			PTFE + Provisional composite			Cotton + Gutta-Percha			Cotton + Provisional composite		
	Mean Count	Proportion %	Prevalence %	Mean Count	Proportion %	Prevalence %	Mean Count	Proportion %	Prevalence %	Mean Count	Proportion %	Prevalence %	Mean Count	Proportion %	Prevalence %
Candida spp.	1.37	6.95	38.33	0.19	1.53	4.16	1.12	15.32	20	0.18	0.66	5.83	1.22	1.79	36.66
V. parvula	0	0	0	0	0	0	0	0	0	0	0	0	1.15	1.69	37.5
T. denticola	3.52	17.87	83.33	0	0	0	0	0	0	0.73	2.69	20.83	0.79	1.16	25
T. forsythia	0	0	0	0	0	0	0	0	0	0.33	1.21	8.33	1.09	1.6	33.33
S. sobrinus	0.28	1.42	8.33	0	0	0	0	0	0	0.27	0.99	8.33	1.72	2.53	54.16
S. sanguinis	0.25	1.26	8.33	0	0	0	0	0	0	1.83	6.74	50	1.33	1.95	41.66
S. salivarius	0.12	0.6	4.16	0	0	0	0	0	0	0.42	1.54	12.5	1.2	1.76	41.66
S. pasteuri[*,†]	0.12	0.6	4.16	3.08	24.83	58.33	3.41	46.64	79.16	0	0	0	3.89	5.73	95.83
S. parasanguinis	0.25	1.26	8.33	0	0	0	0	0	0	0.4	1.47	12.5	1.93	2.84	58.33
S. oralis	0.12	0.6	4.16	0.42	3.38	8.33	0	0	0	0.11	0.4	4.16	2.44	3.59	70.83
S. mutans[*]	0	0	0	0.7	5.64	16.66	0	0	0	0.67	2.47	20.83	3.24	4.77	95.83
S. moorei	0	0	0	0.17	1.37	4.16	0	0	0	1.65	6.08	50	2.24	3.3	66.66
S. mitis	0	0	0	0.17	1.37	4.16	0	0	0	0.46	1.69	12.5	2.44	3.59	75
S. gordonii	0.15	0.76	4.16	0	0	0	0	0	0	2.91	10.7	83.33	2.13	3.13	66.66
S. constellatus	0.25	1.26	8.33	0.15	1.2	4.16	0	0	0	0.12	0.44	4.16	2.15	3.16	66.66
S. aureus	1.7	8.63	50	0.48	3.87	12.5	0	0	0	1.72	6.34	58.33	2.75	4.05	75
P. putida	0	0	0	0.31	2.5	8.33	0	0	0	0.41	1.51	12.5	2.25	3.31	66.66
P. nigrescens[*]	1.01	5.12	16.66	0	0	0	0	0	0	4.17	15.3	95.83	1.59	2.34	54.16
P. micra	0	0	0	0	0	0	0	0	0	0	0	0	0.9	1.32	33.33
P. melaninogenica [†]	3.91	19.85	70.83	0.51	4.11	12.5	2.01	27.49	37.5	0.16	0.58	4.16	1.57	2.31	54.16
P. intermedia	0.28	1.42	8.33	0	0	0	0	0	0	2.32	8.55	58.33	1.13	1.66	41.66
P. gingivalis	0.26	1.32	8.33	0	0	0	0	0	0	1.05	3.87	29.16	1.26	1.85	41.66
P. endodontalis	0.25	1.26	8.33	0	0	0	0	0	0	0.14	0.51	4.16	2.06	3.03	66.66
P. anaerobios	1.61	8.17	50	0	0	0	0	0	0	0.25	0.92	4.16	1.69	2.49	54.16
P. aeruginosa	0	0	0	0	0	0	0	0	0	0.14	0.51	4.16	2.16	3.18	66.66
N. mucosa	0	0	0	0	0	0	0	0	0	0	0	0	2.22	3.27	66.66
M. salivarium[*,†]	0.26	1.32	8.33	4.74	38.2	100	0.77	10.53	16.66	0	0	0	4.09	6.02	95.83
L. casei	0	0	0	0	0	0	0	0	0	0	0	0	1.8	2.65	58.33
K. pneumoniae	0	0	0	0	0	0	0	0	0	1.61	5.93	37.5	1.52	2.23	50
F. periodonticum	0.15	0.76	4.16	0.17	1.37	4.16	0	0	0	2.45	9.03	66.66	0.85	1.25	29.16
F. nucleatum	1.5	7.61	37.5	0	0	0	0	0	0	0.44	1.62	12.5	1.75	2.57	58.33
E. faecalis	0	0	0	0	0	0	0	0	0	0	0	0	1.34	1.97	45.83
E. corrodens	0.28	1.42	8.33	0.48	3.87	12.5	0	0	0	0	0	0	1.65	2.3	54.16
E. coli	0.14	0.71	4.16	0	0	0	0	0	0	0	0	0	1.61	2.37	54.16
C. rectus	0.3	1.52	8.33	0	0	0	0	0	0	0	0	0	1.48	2.18	50
C. gingivalis	0.89	4.52	25	0.31	2.5	8.33	0	0	0	0.58	2.13	16.66	0.8	1.17	29.16
B. fragilis	0.72	3.65	20.83	0.52	4.19	12.5	0	0	0	0.49	1.8	12.5	1.07	1.57	37.5
Aa	0	0	0	0	0	0	0	0	0	1.11	4.09	25	1.36	2	45.83
Total count	19.69	100%		12.40	100%		7.31	100%		27.12	100%		67.86	100%	

*Species with differences in the prevalence between tested groups detected by Kruskal–Wallis followed Bonferroni's multiple comparisons post-tests ($P < 0.05$).
†Species with differences in the proportion between tested groups detected by Kruskal–Wallis followed Bonferroni's multiple comparisons post-tests ($P < 0.05$).
PTFE, Polytetrafluoroethylene; *Aa, Aggregatibacter actinomycetemcomitans*.

components by the abutment screw access channel. Data reported in this controlled experimental study provide a more complete insight on the oral microbiome of screw-retained implant-supported restorations; we not only confirmed previous studies describing the occurrence of microbial leakage by this pathway, but we also extended these findings to a large number of additional species. The characterization of this microbial leakage may provide the basis for further studies helping in the understanding of host–microbe interactions in health and disease.

Conclusions

Within the limitations of this study, we conclude that none of the tested sealing materials prevented microbial leakage through the abutment screw access channel. Moderate to high quantity pathogenic and nonpathogenic microbial species were detected in the inner parts of the implants from all groups. The lowest values of microbial counts, prevalence, and proportions were recorded for polytetrafluoroethylene tape associated with composite resin or gutta-percha; cotton pellet associated with light-polymerized provisional composite presented the highest microbial counts.

Conflict of interest

None of the authors does report any conflict of interest related to the study or products involved. There is no relationship between manufacturer's materials and University of São Paulo (institution in which the study was held).

References

Adrian, E.D., Krantz, W.A., Ivanhoe, J.R. & Turner, K.A. (1991) A silicone obturator for the access canal in an implant-retained fixed prosthesis. *The Journal of Prosthetic Dentistry* **65**: 597.

Albertini, M., López-Cerero, L., O'Sullivan, M.G., Chereguini, C.F., Ballesta, S., Ríos, V., Herrero-Climent, M. & Bullón, P. (2015) Assessment of periodontal and opportunistic flora in patients with peri-implantitis. *Clinical Oral Implants Research* **26**: 937–941.

Augthun, M. & Conrads, G. (1997) Microbial findings of deep peri-implant bone defects. *International Journal of Oral and Maxillofacial Implants* **12**: 106–112.

Cakan, U., Gultekin, P., Guncu, M.B. & Canay, S. (2014) Effect of screw access channel filling materials on uniaxial retentive force of cement-retained implant restorations. *Australian Dental Journal* **59**: 65–69.

Cavalcanti, A.G., Fonseca, F.T., Zago, C.D., Brito, R.B., Jr & França, F.M. (2015) Efficacy of gutta-percha and polytetrafluoroethylene tape to microbiologically seal the screw access channel of different prosthetic implant abutments. *Clinical Implant Dentistry and Related Research*. doi:10.1111/cid.12344.

Cosyn, J., Van Aelst, L., Collaert, B., Persson, G.R. & De Bruyn, H. (2011) The peri-implant sulcus compared with internal implant and suprastructure components: a microbiological analysis. *Clinical Implant Dentistry and Related Research* **13**: 286–295.

Covani, U., Marconcini, S., Crespi, R. & Barone, A. (2006) Bacterial plaque colonization around dental implant surfaces. *Implant Dentistry* **15**: 298–304.

Goiato, M.C., dos Santos, D.M., Santiago, J.F., Jr, Moreno, A. & Pellizzer, E.P. (2014) Longevity of dental implants in type IV bone: a systematic review. *International Journal of Oral and Maxillofacial Surgery* **43**: 1108–1116.

Guindy, J.S., Besimo, C.E., Besimo, R., Schiel, H. & Meyer, J. (1998) Bacterial leakage into and from prefabricated screw-retained implant-borne crowns in vitro. *Journal of Oral Rehabilitation* **25**: 403–408.

Heydecke, G., Zwahlen, M., Nicol, A., Nisand, D., Payer, M., Renouard, F., Grohmann, P., Mühlemann, S. & Joda, T. (2012) What is the optimal number of implants for fixed reconstructions: a systematic review. *Clinical Oral Implants Research* **6**: 217–228.

Jimbo, R. & Albrektsson, T. (2015) Long-term clinical success of minimally and moderately rough oral implants: a review of 71 studies with 5 years or more of follow-up. *Implant Dentistry* **24**: 62–69.

Katsoulis, J., Heitz-Mayfield, L.J., Weibel, M., Hirschi, R., Lang, N.P. & Persson, G.R. (2005) Impact of sample storage on detection of periodontal bacteria. *Oral Microbiology and Immunology* **20**: 128–130.

Kosyfaki, P., del Pilar Pinilla Martín, M. & Strub, J.R. (2010) Relationship between crowns and the periodontium: a literature update. *Quintessence International* **41**: 109–126.

Kwon, T., Bain, P.A. & Levin, L. (2014) Systematic review of short- (5–10 years) and long-term (10 years or more) survival and success of full-arch fixed dental hybrid prostheses and supporting implants. *Journal of Dentistry* **42**: 1228–1241.

Männik, J., Driessen, R., Galajda, P., Keymer, J.E. & Dekker, C. (2009) Bacterial growth and motility in sub-micron constrictions. *Proceedings of the National Academy of Sciences* **106**: 14861–14866.

Moráguez, O.D. & Belser, U.C. (2010) The use of polytetrafluoroethylene tape for the management of screw access channels in implant supported prostheses. *The Journal of Prosthetic Dentistry* **103**: 189–191.

do Nascimento, C., Barbosa, R.E., Issa, J.P., Watanabe, E., Ito, I.Y. & de Albuquerque, R.F., Jr (2009) Use of checkerboard DNA-DNA hybridization to evaluate the internal contamination of dental implants and comparison of bacterial leakage with cast or pre-machined abutments. *Clinical Oral Implants Research* **20**: 571–577.

do Nascimento, C., de Albuquerque, R.F., Jr, Monesi, N. & Candido-Silva, J.A. (2010) Alternative method for direct DNA probe labeling and detection using the checkerboard hybridization format. *Journal of Clinical Microbiology* **48**: 3039–3040.

do Nascimento, C., Miani, P.K., Pedrazzi, V., Muller, K. & de Albuquerque, R.F., Jr (2012) Bacterial leakage along the implant-abutment interface: culture and DNA Checkerboard hybridization analyses. *Clinical Oral Implants Research* **23**: 1168–1172.

do Nascimento, C., Monesi, N., Ito, I.Y., Issa, J.P. & de Albuquerque, R.F., Jr (2011) Bacterial diversity of periodontal and implant-related sites detected by the DNA Checkerboard method. *European Journal of Clinical Microbiology and Infectious Diseases* **30**: 1607–1613.

Neilands, J., Wickström, C., Kinnby, B., Davies, J.R., Hall, J., Friberg, B. & Svensäter, G. (2015) Bacterial profiles and proteolytic activity in peri-implantitis versus healthy sites. *Anaerobe* **35**: 28–34.

Palma-Carrió, C., Maestre-Ferrín, L., Peñarrocha-Oltra, D., Peñarrocha-Diago, M.A. & Peñarrocha-Diago, M. (2011) Risk factors associated with early failure of dental implants. A literature review. *Medicina Oral Patologia Oral y Cirurgia Bucal* **16**: 514–517.

Park, S.D., Lee, Y., Kim, Y.L., Yu, S.H., Bae, J.M. & Cho, H.W. (2012) Microleakage of different sealing materials in access holes of internal connection implant systems. *The Journal of Prosthetic Dentistry* **108**: 173–180.

Passos, S.P., Gressler May, L., Faria, R., Özcan, M. & Bottino, M.A. (2013) Implant-abutment gap versus microbial colonization: clinical significance based on a literature review. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **101**: 1321–1328.

Pjetursson, B.E., Asgeirsson, A.G., Zwahlen, M. & Sailer, I. (2014) Improvements in implant dentistry over the last decade: comparison of survival and complication rates in older and newer publications. *International Journal of Oral and Maxillofacial Implants* **29**: 308–324.

Pjetursson, B.E., Thoma, D., Jung, R., Zwahlen, M. & Zembic, A. (2012) A systematic review of the survival and complication rates of implant-supported fixed dental prostheses (FDPs) after a mean observation period of at least 5 years. *Clinical Oral Implants Research* **6**: 22–38.

Quirynen, M., Bollen, C.M., Eyssen, H. & van Steenberghe, D. (1994) Microbial penetration along the implant components of the Brånemark system. An in vitro study. *Clinical Oral Implants Research* **5**: 239–244.

Quirynen, M. & van Steenberghe, D. (1993) Bacterial colonization of the internal part of two stage implants. An in vivo study. *Clinical Oral Implants Research* **4**: 158–161.

Quirynen, M., Vogels, R., Pauwels, M., Haffajee, A.D., Socransky, S.S., Uzel, N.G. & van

Steenberghe, D. (2005) Initial subgingival colonization of 'pristine' pockets. *Journal of Dental Research* **84**: 340–344.

Sakka, S., Baroudi, K. & Nassani, M.Z. (2012) Factors associated with early and late failure of dental implants. *Journal of Investigative and Clinical Dentistry* **3**: 258–261.

Socransky, S.S., Haffajee, A.D., Cugini, M.A., Smith, C. & Kent, R.L., Jr. (1998) Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology* **25**: 134–144.

Socransky, S.S., Smith, C., Martin, L., Paster, B.J., Dewhirst, F.E. & Levin, A.E. (1994) "Checkerboard" DNA-DNA hybridization. *BioTechniques* **17**: 788–792.

Tarica, D.Y., Alvarado, V.M. & Truong, S.T. (2010) Survey of United States dental schools on cementation protocols for implant crown restorations. *The Journal of Prosthetic Dentistry* **103**: 68–79.

Taylor, R.C., Ghoneim, A.S. & McGlumphy, E.A. (2004) An esthetic technique to fill screw retained fixed prostheses. *Journal of Oral Implantology* **30**: 384–385.

Weininger, B., McGlumphy, E. & Beck, M. (2008) Esthetic evaluation of materials used to fill access holes of screw-retained implant crowns. *Journal of Oral Implantology* **34**: 145–149.

Yamane, K., Ayukawa, Y., Takeshita, T., Furuhashi, A., Yamashita, Y. & Koyano, K. (2013) Bacterial adhesion affinities of various implant abutment materials. *Clinical Oral Implants Research* **24**: 1310–1315.

Zhuang, L.F., Watt, R.M., Mattheos, N., Si, M.S., Lai, H.C. & Lang, N.P. (2014) Periodontal and peri-implant microbiota in patients with healthy and inflamed periodontal and peri-implant tissues. *Clinical Oral Implants Research* **27**: 13–21.