CLINICAL ORAL IMPLANTS RESEARCH

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Microbiological assessment of the implant-abutment interface in different connections: cross-sectional study after 5 years of functional loading

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Abstract

Objective: To evaluate the bacterial microflora present inside the implant connection and in the peri-implant sulcus fluid of healthy implants, and to analyze the relationships between these harboring sites for four different implant systems after at least 5 years of functional loading.

Materials and methods: A cross-sectional study was performed involving 40 patients treated with metal-ceramic cemented bridges supported by at least two healthy implants functionally loaded for 5 years. Four different implant-abutment connections were studied: external hexagon (control group), double internal hexagon (test group 1), internal hexagon with external collar (test group 2), and conical connection (test group 3). Samples for microbiological analysis were obtained from three types of sites: peri-implant sulci, connections' inside and abutments surface and, as control, gingival sulci of neighboring teeth. Quantitative real-time PCR was carried out for Total Bacterial Count and for 10 microorganisms: *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythensis, Treponema denticola, Prevotella intermedia, Peptostreptococcus micros, Fusobacterium nucleatum, Campylobacter rectus, Eikenella corrodens,* and *Candida albicans.* The response variables were percentage of positive sites and absolute bacterial load. The relations of the response variables with the type of connection and of sampling site were assessed using generalized estimating equations.

Results: Regarding the analysis of positivity to bacteria in the peri-implant sulcus no significant differences were observed. Analyzing the connection's inside, none of the connection designs had the capacity to prevent microbiological leakage through the implant/abutment microgap. Test group 3 presented the lowest mean values for red complex bacteria and control group the highest, although differences were non-significant. Statistical significance was only reached for *Treponema denticola* in the bacterial load analysis inside the connection. Test groups 1 and 2 yielded lower values for orange complex bacteria but only for *Peptostreptococos micros* the differences resulted significant. Test groups 2 and 3 had significantly lower total bacterial counts in the peri-implant sulcus and inside the connection.

Conclusions: Outcomes suggested that all the analyzed connections resulted contaminated after 5 years of functional loading. However, the connection design might influence bacterial activity levels qualitatively and quantitatively, especially inside the implant connection.

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Two-piece implants unavoidably present a micro-gap between the implant and the abutment. Bacterial leakage through this micro-gap at the implant-abutment interface (IAI) and colonization of the connection's inner portion is well-proved (Do Nascimento et al. 2012). These spaces, once early colonized, may constitute a bacterial reservoir. It could subsequently contaminate a fixture's surroundings and interfere with peri-implant tissues health (Do Nascimento et al. 2008; Tesmer et al. 2009; Teixeira et al. 2011). The presence of a micro-gap, and thus a reservoir of bacteria, when in close relation to bone, may have a role in the development of peri-implant tissues inflammation and bone loss (Hermann et al. 2000; Piattelli et al. 2003; Broggini et al. 2006). In fact, this micro-gap at the IAI has been suggested as one of the possible etiological factors of early crestal bone loss around implants (Oh et al. 2002). The inside of the connection is an environment with low oxygen concentration and away from

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the inflammatory defensive response of the peri-implant tissues, thus providing a perfect environment for the development of anaerobic bacteria that may be particularly related with peri-implant pathology (Persson et al. 1996). The long-term role of this bacterial colonization must be considered in the maintenance of healthy peri-implant tissues (Passos et al. 2013). A recent systematic review concluded that while the positioning of the microgap may influence crestal bone level changes, the impact of the implant-abutment connection lacks documentation (Schwarz et al. 2013).

The potential colonization of the internal connection through the implant-abutment micro-gap is probably related to multifactorial conditions, i.e., the precision fit between the implant components, which is associated with the implant system design (Tesmer et al. 2009; Steinebrunner et al. 2005); the torque used to connect the components (Gross et al. 1999); the repeated screw loosening and re-tightening (Do Nascimento et al. 2009); and the loading forces when the implants are in function (Koutouzis et al. 2012; Kitagawa et al. 2011).

Different connections have been compared regarding their mechanical stability. Higher stability under loading conditions has been reported for different internal connections compared to external connections (Kitagawa et al. 2005; Maeda et al. 2006; Da Silva et al. 2010). According to Salvi & Lang (2001), under functional loading, the mechanical instability of the external connection promotes micro-movements of the abutment. Differently from other connections, the Morse-cone connection locks the implant/abutment system because of the friction between the external wall of the abutment and internal wall of the implant (Salvi & Lang 2001).

A recent systematic review (Schmitt et al. 2013) indicated that conical and non-conical abutments showed sufficient resistance to maximal bending forces and fatigue loading. However, conical abutments showed superiority in terms of seal performance, microgap formation, torque maintenance, and abutment stability.

Although these features could theoretically lead to better clinical results over time, no evidence is available (Schmitt et al. 2013). Moreover, consensus does not exist even within *in vitro* studies (Saidin et al. 2012).

The evidence regarding differences in microbial penetration of the implant/abutment micro-gap with different connection designs is very limited and mostly based on *in vitro* studies. Many of these studies claim that conical connections have smaller gaps and thus are superior to other internal and external screw-retained connections in limiting bacterial leakage (Tesmer et al. 2009; D'Ercole et al. 2011; Koutouzis et al. 2011; Assenza et al. 2012; Tripodi et al. 2012), while other authors conclude that differences are non-significant (Teixeira et al. 2011). In any case, no endosseous dental implant system can currently provide a complete seal at the IAI, occurring bacterial leakage irrespective of the type of connection (Passos et al. 2013; Schwarz et al. 2013).

Loading forces on the prosthetic components may induce implant-abutment system bending or micromovement. This has been associated with the increase in the microgap and a "pump effect" between the inside of the implant and the peri-implant tissues (Merz et al. 2000; Steinebrunner et al. 2005). Conical systems appear to be superior to internal and external connections in limiting this inconvenient (Merz et al. 2000). Accordingly, a few *in vitro* studies have analyzed microleakage under dynamic loading (Steinebrunner et al. 2005; Koutouzis et al. 2011, 2012) and showed that conical connections were superior to internal connection in preventing bacterial leakage through the IAI.

However, irrespectively of the complexity of models built to simulate chewing and the intraoral environment, reproducing *in vitro* clinical conditions remains impossible.

In fact, available comparative clinical studies have only analyzed success rate and bone level changes of implants with different connection. In these studies implant success rates did not vary between conical and non-conical implant-abutment connection systems but less marginal bone loss was reported for conical connection systems (Bilhan et al. 2010; Pieri et al. 2011). However, no clinical study has been reported regarding bacterial permeability of different implant-abutment connections under *in vivo* functional conditions, and thus a lack of evidence remains.

The aims of the present study were to evaluate the bacterial microflora present inside the implant connection and in the peri-implant sulcus fluid of healthy implants, and to analyze the relationships between these harboring sites of four different implant systems after at least 5 years of functional loading. The undisturbed subgingival microbiota of neighboring healthy teeth in the same individuals served as controls.

Material and methods

Study design

A cross-sectional study was performed following the principles outlined in the Declaration of Helsinki of patients previously treated with dental implants. Patients were recruited between September 2011 and July 2013 at four private specialist centers (Rome, Viareggio, Padua, Vicenza, Italy), and were required to sign a consent form after being informed about the study.

Four different types of implant-abutment connections (Fig. 1) were studied in four groups of patients: external hexagon (; Control Group [CG]; BIOMET3i®, Palm Bring, US), double internal hexagon (Test Group 1 [TG1]; Certain®, BIOMET3i®, Palm Bring, US), internal hexagon with external collar (Test Group 2 [TG2]; Premium-Kohno®, Sweden&Martina®, Padua, Italy), and conical connection (Test Group 3 [TG3]; Conical Seal Design®, ASTRA TECH Implant System®, Mannheim, Germany). Ten patients with fixed metal-ceramic cemented bridges supported by at least two healthy implants functionally loaded for 5 years, neighboring healthy teeth and with the necessity to have the prosthesis removed to be repaired or substituted for a new one were included per group. Subject and study site inclusion and exclusion criteria are detailed in Table 1.

All patients had participated in maintenance programs with routine control visits including oral professional prophylaxis every 6–12 months since their implants had been placed.

Biotype (thick/thin), gender and age were registered for each patient, and position for each implant.

Microbiological sampling

Sampling for microbiological analysis from all groups was performed by a single researcher. Samples were obtained from three types of sites in each patient in the following order: (1) the peri-implant sulcus (PIS) of each implant, (2) the gingival sulcus of neighboring teeth (GS), (3) and the connection's inside and the abutment surface (CIAS) of each implant.

Sampling was performed using GUIDOR® Perio-Implant Diagnostic Test (Sunstar Iberia S.L.U, Barcelona, Spain) kits, consisting in five sterile absorbent paper tips and a 2 ml sterile empty Eppendorf tube. Prior to subgingival plaque sampling, supragingival plaque was eliminated from implants and teeth using a curette or cotton roll, without penetrating the gingival or peri-implant sulcus.

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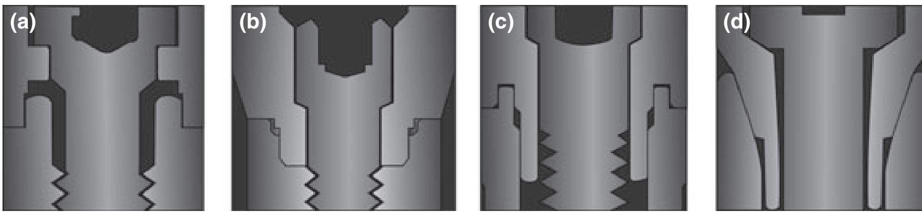

Fig. 1. Drawing showing the analyzed connections. (a) control group; (b) test group 1; (c) test group 2; (d) test group 3.

***Table 1.* Subject and study site inclusion and exclusion criteria**

Subject inclusion criteria
Presence of a fixed metal-ceramic cemented bridge supported by at least two implants having to be removed to be repaired or substituted for a new one
Healthy peri-implant tissues: absence of bleeding on gentle probing (<0.25 N), PPD ≤ 5 mm and absence of radiographic bone loss assessed in paralleled periapical radiographs (Lang & Berglundh 2011)
Uneventful functional loading for at least 5 years; the bridge must have not been removed during this time
Age > 18 years
Specific subject and site exclusion criteria
Presence of active periodontal or peri-implant pathology in any site of the mouth (diagnostic criteria: bleeding on gentle probing (<0.25 N) and PPD > 3 mm in teeth and > 5 mm in implants)
Use of antimicrobials during the 3 months prior to the study
Smokers (at present or during the 12 months prior to the study)
Pregnant and lactating patients
Patients with a history of Bisphosphonate therapy
Patients with known systemic disease or metabolic disorders (e.g. HIV, diabetes) and with medications (e.g. high dose steroid therapy, systemic treatment with tetracycline or tetracycline analogs, bone therapeutic levels of fluorides, bisphosphonates, medication affecting bone turnover, or any investigational drug) that are detrimental to soft tissue and/or bone healing.
Note: topical application of steroids and steroid application trough inhalation is not an exclusion criterion
Patients who had malignant diseases or other diseases treated with radiotherapy or chemotherapeutic agents ("chemotherapy") during the past 5 years
Patients with a history of head and neck radiation treatment due to certain medical conditions
Severe alcohol or drug users
Patients refusing to sign an informed consent document or to participate in the study

Cotton rolls were used for relative isolation and the sampling sites were dried with an air pistol. The paper tips were inserted in the gingival or peri-implant sulci for 30 s.

To collect the samples of the implant connection, prostheses and abutments were carefully removed, trying to avoid contamination. One drop of RNA-and DNA-free water [Water Molecular Biology Reagent (SIGMA) code W4502] was placed inside the implant connection and three paper tips were inserted for 30 s. The connection surface of the abutment was wetted with a drop of RNA-and DNA-free water and smeared with two paper tips.

The paper tips were placed into the Eppendorf tubes and were sent for microbiological analysis to the laboratory Institut Clinident SAS (Aix en Provence, France) in the provided mailing envelopes.

Quantitative real-time PCR assays

Quantitative real-time PCR was carried out for Total Bacterial Count (TBC) and for 10 pathogens: *Aggregatibacter actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg), Tannerella forsythensis (Tf), Treponema denticola (Td), Prevotella intermedia (Pi), Peptostreptococcus micros (Pm), Fusobacterium nucleatum (Fn), Campylobacter rectus (Cr), Eikenella corrodens (Ec)* , and *Candida albicans (Ca).*

Quantitative real-time PCR assays were performed in a volume of 10 μl composed of 1 × QuantiFast® SYBR® Green PCR (Qiagen, Germany), 2 μl of DNA extract and 1 μM of each primer. The species-specific PCR primers used in this study were provided by Institut Clinident SAS (Aix en Provence, France) and manufactured by Metabion GmbH (Martinsried, Germany). The bacterial primers used are derived from previously published ribosomal 16S sequences (Slots et al. 1995; Sakamoto et al. 2001; Martin et al. 2002; Suzuki et al. 2005) and have been adapted to the real-time PCR conditions. *Candida albicans* primers used in this study are derived from ribosomal 18S/28S sequences.

Assays were carried out on the Rotor-Gene® Q thermal cycling system (Qiagen, Germany) with the following program: 95°C for 5 min, followed by 40 cycles of 10 s at 95°C, 10 s at 60°C, and 35 s at 72°C. A final melt curve analysis (70 to 95°C in 1°C steps for 5 s increments) was done. Fluorescence signals were measured every cycle at the end of the extension step and continuously during the melt curve analysis. The resulting data were analyzed using Rotor-Gene® Q Series software (Qiagen, Germany).

Serial dilutions of bacterial standard DNA provided by Institut Clinident SAS were used in each reaction as external standards for absolute quantitation of the targeted bacterial pathogens. Standard bacterial strains used for standard DNA production were obtained from DSMZ (Braunschweig, Germany), CIP Collection of Institut Pasteur (Paris, France) or from BCMM/LMG Bacteria Collection (Ghent, Belgium): *Aa* (DSM No. 8324), *Pg* (DSM No. 20709), *Tf* (CIP No. 105220), *Td* (DSM No. 14222), *Pi* (DSM No. 20706), *Pm* (DSM No. 20468), *Fn* (DSM No. 20482), *Cr* (LMG No. 18530), *Ec* (DSM No. 8340). A *Ca* standard DNA *(DSM No. 6659)* was also used as external standard for identification and semi-quantitation.

Statistical analysis

Homogeneity of the sample by type of connection was verified using Kruskal-Wallis test for age and using Goodman and Kruskal's Tau test for sex and biotype.

The response variables of the study were percentage of positive sites (PPS) and absolute bacterial load (BL: number of bacteria) for the different analyzed pathogens. PPS for individual pathogenic species and for combinations of species belonging to the red and orange complexes (Socransky et al. 1998) were studied for PIS and CIAS. BL was analyzed for individual pathogens and TBC in PIS, CIAS and also in GS, which served as control. Differences in BL-GS between groups were analyzed using Kruskal-Wallis test. Original figures for BL reached the range of 10^6 and were logarithmic transformed to simplify the description and interpretation of results. The comparison between BL-CIAS and BL-PIS can be interpreted as an estimate of the sealing capacity of the IAI. To do so, a 'standardized bacterial contamination index' (SBCI) was defined as the ratio $\frac{log_{10}\,\mathrm{BL{-}CIAS}}{log_{10}\,\mathrm{BL{-}PIS}}$. The SBCI was another response variable for those pathogens with enough PPS to perform statistical analysis.

The relationships of these response variables with the type of connection and the type of sampling site were assessed using

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generalized estimating equations (GEE) models to correct the effect of the intra-subject correlation. Wald's χ^2 test was used to evaluate the hypothesis of homogeneous contamination by bacteria in the different connection types.

The proposed EEG model had a power of 0.84 to detect an effect size of f = 0.40 in the level of bacterial load compared between connection types, assuming a 95% confidence for the studied sample of $n = 80$ implants. The significance level used for the analysis was 5% ($\alpha = 0.05$).

Table 2. **Descriptive statistics for the logarithm of the bacterial load (BL) in the gingival sulcus of neighboring teeth for each group (mean±SD). Results for Kruskal-Wallis test**

	Study group				
Log (Bacteria)	CG	TG1	TG2	TG3	*P*-value (KW)
Log (Aa)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.000
Log (Pg)	3.16 ± 3.11	2.64 ± 2.29	1.94 ± 2.72	2.03 ± 3.14	0.823
Log (Tf)	1.63 ± 2.80	1.13 ± 2.03	3.04 ± 1.98	1.59 ± 2.51	0.303
Log (Td)	0.88 ± 2.34	0.80 ± 2.22	2.32 ± 2.51	1.02 ± 2.50	0.394
Log (Pi)	1.72 ± 3.04	1.18 ± 2.04	2.49 ± 2.67	4.37 ± 2.28	0.202
Log (Pm)	4.48 ± 2.18	4.75 ± 0.64	4.04 ± 0.95	4.08 ± 2.09	0.555
Log (Fn)	5.55 ± 0.72	3.60 ± 3.32	5.06 ± 079	5.60 ± 0.91	0.542
Log (Cr)	3.95 ± 2.77	2.68 ± 2.85	3.70 ± 2.32	3.67 ± 2.90	0.307
Log (Ec)	3.39 ± 3.26	3.02 ± 3.15	3.51 ± 2.27	3.22 ± 2.59	0.805
Log (Ca)	0.96 ± 2.53	0.00 ± 0.00	0.00 ± 0.00	0.55 ± 1.36	0.621
Log (TBC)	8.28 ± 0.82	7.51 ± 1.08	7.03 ± 0.81	7.75 ± 0.83	0.150

Results

Description of the study sample

During the study period, 60 screened patients fulfilled the inclusion criteria. Twenty patients were excluded: four patients presented with gingivitis, two had taken systemic antibiotics during the 3 months prior to the microbiological sampling, 12 patients were or had been smokers during the 12 months prior to the study, one patient was pregnant and one patient refused to participate in the study. The final sample consisted of forty patients (23 male and 17 female; mean age 69.9 ± 16.7 years) – 10 patients for each type of connection – and 80 analyzed implants. Biotypes were almost balanced between thin ($n = 18$) and thick ($n = 22$). Homogeneity tests yielded no significant difference between study groups in terms of age ($P = 0.339$), gender ($P = 0.597$), and biotype ($P = 0.315$). Furthermore, no statistically significant difference was observed in bacterial load of neighboring teeth between study groups. Table 2 details the descriptive and comparative analysis of BL-GS.

Table 3. **Descriptive statistics (mean ± SD) for the percentage of positive sites (PPS) to individual bacteria and combinations of red and orange complexes' bacteria inside the connection and in the abutment surface (CIAS) and in the peri-implant sulcus (PIS) for each group. Results for Wald's Chi[2] test of the estimated GEE model**

	Type of sampling site and study group									
	PIS					CIAS				
Bacteria	CG	TG1	TG2	TG3	*P*-value (Wald)	CG	TG1	TG2	TG3	*P*-value (Wald)
Aa	15	0	0	0	0.432	5	0	0	0	0.480
Pg	75	100	55	60	0.682	50	60	30	10	0.062†
Tf	65	100	100	70	0.173	60	40	45	20	0.357
Td	50	65	55	20	0.355	50	25	25	0	0.053†
Pi	75	70	80	80	0.962	40	35	25	60	0.201
Pm	100	100	90	100	0.506	95	65	75	100	0.047*
Fn	100	80	100	100	0.129	100	85	90	90	0.427
Cr	75	65	80	80	0.979	40	5	30	50	0.055†
Ec	50	65	45	60	0.892	20	20	5	10	0.617
Ca	15	35	0	10	0.428	5	15	0	20	0.261
Pg+Tf	65	100	55	50	0.886	40	35	25	0	0.116
Pg+Td	40	65	45	10	0.328	35	20	15	0	0.093†
Tf+Td	25	65	55	20	0.249	50	25	25	0	0.053†
Red complex (Pg+Tf+Td)	25	65	45	10	0.270	35	20	15	0	0.093†
Pi+Pm	75	70	80	80	0.201	40	35	25	60	0.201
Pi+Fn	75	50	80	80	0.560	40	35	25	50	0.560
Pm+Fn	100	80	90	100	0.183	95	55	70	90	0.153
Orange complex (Pi+Pm+Fn)	75	50	80	80	0.560	40	35	25	50	0.560

†$P < 0.1$ (tendency to significance); *$P < 0.05$.

Analysis of the percentage of positive sites

Descriptive and comparative statistics of percentage of positive sites (PPS)-PIS and PPS-CIAS for the different pathogens and for combinations of red and orange complexes' bacteria are detailed in Table 3.

Regarding PPS-PIS (Fig. 2), no significant difference between groups was observed for any of the studied pathogens or for combinations of red and orange complexes' bacteria.

Regarding PPS-CIAS, TG3 presented the lowest mean values and CG the highest for red complex bacteria. Differences did not reach statistical significance but showed a tendency to significance for *Pg, Td* and the combinations *Pg+Td,Tf+Td* and *Pg+Tf+Td* (Fig. 3a). TG1 and TG2 yielded lower positivity values for orange complex bacteria and their combinations (Fig. 3b) but only for *Pm* and *Pm+Pi* were differences statistically significant.

Analysis of bacterial load

Descriptive and comparative statistics of BL-PIS and BL-CIAS for the individual pathogens and for Total Bacterial Load are detailed in Table 4.

Regarding the analysis of BL-PIS (Fig. 4), TG3 showed the lowest values for red complex bacteria. Differences did not reach statistical significance but showed tendency to significance for *Pg* and *Tf*. Significant differences were only observed for *Pm* and *Ca,* for which the mean BL-PIS values were lowest in TG2.

Fig. 5 represents graphically the BL-CIAS analysis. TG3 was associated with lower mean values for red complex bacteria but statistical significance was only reached for *Td*. For this bacterial species, TG1 and TG2 gave higher mean BL than TG3 but lower than CG. For *Pg* differences tended to significance, and again TG3 yielded the lowest BL values. In this case, TG2 had a lower average value than TG1 and external hexagon, which showed a similar behavior. Regarding the orange complex, TG1 and TG2 showed significantly lower BL-CIAS values for *Fn* and *Pm* than CG and TG3.

Significant differences between connections were also observed in TBC-PIS and TBC-CIAS, being values lower for TG2 and TG3 than for CG and TG1.

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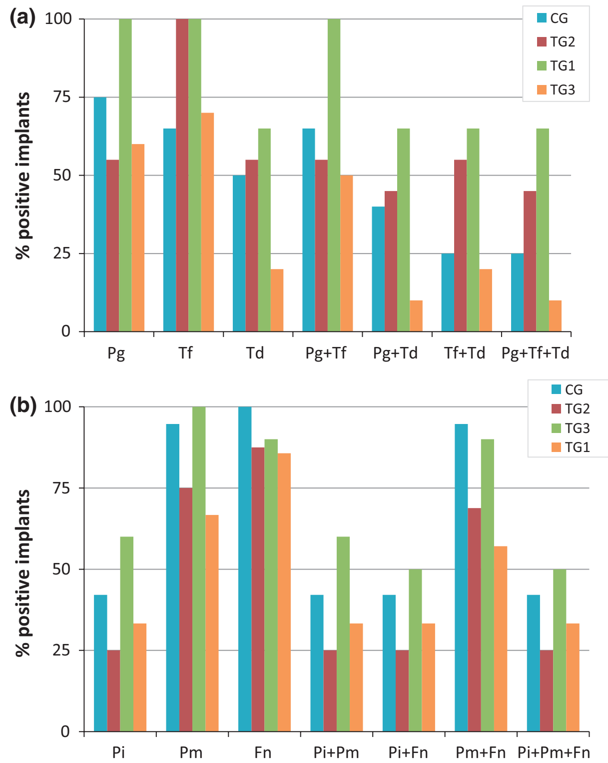

Fig. 2. Percentage of implants showing positivity (PPS) inside the connection and on the abutment surface (CIAS) to (a) red complex bacteria and (b) orange complex bacteria and their combinations

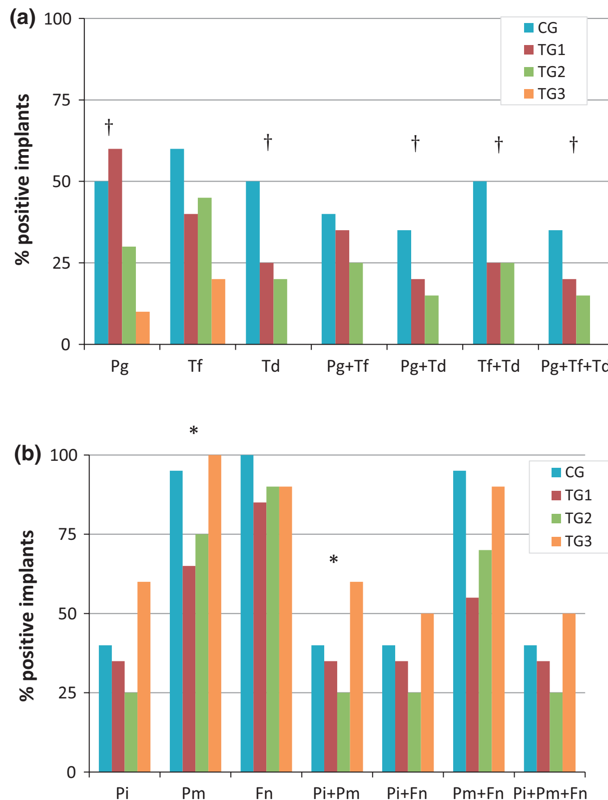

Fig. 3. Percentage of implants showing positivity (PPS) inside the connection and on the abutment surface (CIAS) to (a) red complex bacteria and (b) orange complex bacteria and their combinations ($^{\dagger}P < 0.1$ (tendency to significance); $^{*}P < 0.05$)

Analysis of the 'standardized bacterial contamination index'

The statistical analysis for 'standardized bacterial contamination index' (SBCI) is detailed in Table 5 and graphically represented in Fig. 6. *Aa* and *Ca* were not studied because the number of positive sites for this bacteria was insufficient.

The descriptive analysis shows that mean SBCI values are <1 in most cases, meaning that BL-PIS is usually higher than BL-CIAS.

Regarding the red complex, mean SBCI values were significantly different between groups for *Pg* and *Td* and differences tended to significance for *Tf*. For these bacteria, TG3 showed the lowest mean SBCI values. Statistically significant differences were obtained also for two orange complex bacteria - *Pm* and *Fn* -, but in this case TG1 and TG2 yielded lower mean SBCI values than CG and TG3. Differences were also significant for *Cr*, for which TG1 obtained the lowest mean SBCI value, and for *Ec*, for which the mean BL-CIAS value and, thus, the mean SBCI value were equal to 0 for TG2.

Discussion

Microbial penetration through the micro-gap that inevitably exists between implants and abutments and colonization of the connection's inner portion are demonstrated by *in vitro* (Teixeira et al. 2011; Assenza et al. 2012) and *in vivo* studies (Persson et al. 1996; Orsini et al. 2000). A bacterial reservoir may establish inside the implant that, in the long term, could seriously affect the health of peri-implant tissues (Do Nascimento et al. 2008; Teixeira et al. 2011).

Up to now, no implant system or connection design has been able to provide a perfect sealing at the IAI (Passos et al. 2013; Schwarz et al. 2013), although conical connections seem to be superior in reducing bacterial leakage according to *in vitro* studies (Tesmer et al. 2009; D'Ercole et al. 2011). The present study aimed at comparing the sealing capacity to microbial leakage of four different implant connections under *in vivo* conditions.

Three response variables were established to study the sealing capacity to microbial leakage of the different connections. The positivity analysis (PPS to individual pathogenic species or to combination of species) provided a qualitative approach, while the bacterial load analysis provided a pure quantitative approach. A third response variable, the 'standardized bacterial contamination index', was derived to compare the sealing capacities of

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Table 4. **Descriptive statistics (mean±SD) for the logarithm of the bacterial load (BL) inside the connection and on the abutment surface (CIAS) and in the peri-implant sulcus (PIS). Results for Wald's Chi^2 test of the estimated GEE model**

Log (Bacteria)	CIAS CG	CIAS TG1	CIAS TG2	CIAS TG3	CIAS *P*-value (Wald)	PIS CG	PIS TG1	PIS TG2	PIS TG3	PIS *P*-value (Wald)
Log (Aa)	0.70 ± 1.98	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.670	0.35 ± 1.52	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.795
Log (Pg)	4.18 ± 2.76	4.71 ± 1.20	3.10 ± 2.96	3.06 ± 2.71	0.054†	2.37 ± 2.69	2.48 ± 2.33	1.30 ± 2.10	0.36 ± 1.13	0.060†
Log (Tf)	3.63 ± 3.06	5.57 ± 0.95	4.68 ± 0.78	3.24 ± 2.29	0.070†	3.05 ± 2.77	1.71 ± 2.27	1.89 ± 2.26	0.75 ± 1.57	0.164
Log (Td)	2.80 ± 3.05	3.87 ± 3.41	2.86 ± 2.73	1.01 ± 2.13	0.386	2.58 ± 2.87	1.08 ± 2.00	1.23 ± 2.21	0.00 ± 0.00	0.028*
Log (Pi)	4.87 ± 3.24	4.56 ± 3.97	4.13 ± 2.45	4.82 ± 2.77	0.955	2.22 ± 2.78	1.83 ± 2.69	1.24 ± 2.22	2.89 ± 2.51	0.219
Log (Pm)	5.53 ± 1.41	5.80 ± 0.29	4.09 ± 1.59	4.87 ± 1.07	0.005**	5.13 ± 1.73	3.32 ± 2.63	2.96 ± 2.02	4.93 ± 0.49	0.022*
Log (Fn)	6.13 ± 0.88	4.31 ± 3.75	5.48 ± 0.047	5.66 ± 0.96	0.212	5.33 ± 1.01	4.23 ± 1.97	3.60 ± 1.65	4.56 ± 1.74	0.008**
Log (Cr)	4.59 ± 2.89	3.26 ± 2.85	4.10 ± 2.38	4.32 ± 2.31	0.900	2.23 ± 2.75	0.27 ± 1.22	1.62 ± 2.50	2.51 ± 2.68	0.109
Log (Ec)	3.01 ± 3.29	3.56 ± 3.08	1.99 ± 2.45	2.71 ± 2.36	0.785	1.06 ± 2.15	0.85 ± 1.83	0.26 ± 1.02	0.47 ± 1.50	0.524
Log (Ca)	0.48 ± 1.35	1.93 ± 3.35	0.00 ± 0.00	0.50 ± 1.58	0.031*	0.26 ± 1.15	1.00 ± 2.52	0.00 ± 0.00	1.02 ± 2.21	0.263
Log (Total)	8.71 ± 0.84	9.03 ± 3.46	7.75 ± 0.35	7.89 ± 0.77	<0.001***	8.04 ± 0.98	7.38 ± 0.86	6.33 ± 0.93	6.92 ± 0.60	<0.001***

$^{\dagger}P < 0.1$ (tendency to significance); * $P < 0.05$; *** $P < 0.001$.

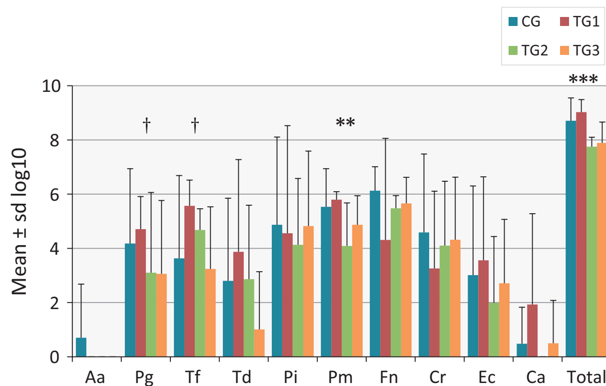

Fig. 4. Logarithm of the bacterial load (Log_{10} (BL)) for individual pathogens and for total bacterial load in the peri-implant sulcus (PIS) ($^{\dagger}P < 0.1$ (tendency to significance); $**P < 0.01$; $***P < 0.001$).

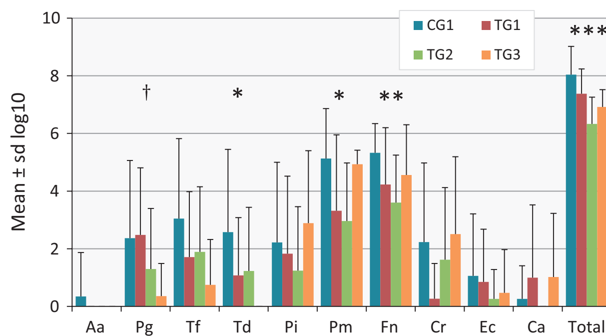

Fig. 5. Logarithm of the bacterial load (Log_{10} (BL)) for individual pathogens and for total bacterial load inside the connection and on the abutment surface (CIAS) ($^{\dagger}P < 0.1$ (tendency to significance); $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

the different connections considering the *in vivo* conditions.

In *in vitro* studies, one of the two environments [either the inside (Kitagawa et al. 2011) or the outside (Assenza et al. 2012) of the implant] is sterile at the beginning. However, under *in vivo* conditions, the presence of bacteria contaminating the internal portion of the connection is unavoidable from the beginning (Persson et al. 1996). Therefore, after long term functional loading, two colonized environments are present separated by the IAI. The SBCI is defined as a ratio that considers the BL in both type of harboring sites (CIAS and PIS). As this ratio gets closer to 1, this indicates that the two environments are more similar in terms of bacterial population. This, in turn, suggests that a worse sealing is being provided by the implant connection system.

Differences in the peri-implant microbiota might occur as the result of various characteristics associated with the implant system (i.e., material, coating, roughness, shape) (Quirynen et al. 2002; Papaspyridakos et al. 2012). However, different studies could not relate the presence of particular microorganisms in the PIS to particular implant systems (Alcoforado et al. 1991; Mombelli et al. 1995; Keller et al. 1998; Lee et al. 1999; Renvert et al. 2008; Tabanella et al. 2009).

Contrarily, in the present study several statistically significant differences were observed. At the end of the study, data suggested that all the involved connections resulted microbiologically contaminated. However, while the external hexagon (CG) presented the worst results, internal (TG1 and TG2) and conical (TG3) connections showed significantly better results.TG3 had significantly lower mean values for the three response variables when considering bacteria belonging to Socransky's red complex (Socransky et al. 1998). On the contrary, TG1 and, specially, TG2 yielded lower mean values for the three response variables in terms of orange complex bacteria. Finally, TG2 and TG3 were superior in terms of reducing Total Bacterial Load both in CIAS and PIS.

Study design and technical conditions of the present investigation limit the conclusions that can be drawn. The sample size for each group was small; cases of healthy implants with at least 5 years of follow-up in which prosthesis removal was justified were not frequent. To overcome this drawback four different study centers were involved. This introduced a new variable: the prosthetic treatments were performed by different operators. Procedures within the prosthetic phase, have been demonstrated to influence bacterial leakage (Gross et al. 1999; Do Nascimento et al. 2009).

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Table 5. **Descriptive statistics (mean ± sd) for the 'standardized bacterial contamination index' (SBCI). Results for Wald's Chi^2 test of the estimated GEE model**

	Study group								
	CG		TG1		TG2		TG3		
Bacteria	*N*	Mean±SD	*N*	Mean ± SD	*N*	Mean±SD	*N*	Mean±SD	*P*-value Wald
Pg	15	0.61 ± 0.44	20	0.41 ± 0.41	11	0.35 ± 0.44	12	0.13 ± 0.30	0.010*
Tf	13	0.63 ± 0.43	20	0.37 ± 0.49	20	0.38 ± 0.45	14	0.24 ± 0.41	0.052†
Td	10	0.79 ± 0.49	13	0.29 ± 0.43	11	0.42 ± 0.51	4	0.00 ± 0.00	<0.001***
Pi	15	0.49 ± 0.43	14	0.45 ± 0.44	16	0.18 ± 0.37	16	0.45 ± 0.44	0.108
Pm	20	1.05 ± 0.42	20	0.58 ± 0.47	18	0.64 ± 0.44	20	1.03 ± 0.25	0.020*
Fn	20	0.92 ± 0.21	14	0.71 ± 0.26	20	0.65 ± 0.29	20	0.82 ± 0.33	0.038*
Cr	15	0.54 ± 0.46	13	0.08 ± 0.29	16	0.33 ± 0.46	16	0.64 ± 0.45	<0.001***
Ec	10	0.33 ± 0.47	13	0.22 ± 0.38	9	0.00 ± 0.00	12	0.19 ± 0.45	0.010*
TBC	20	0.95 ± 0.18	20	0.82 ± 0.12	20	0.82 ± 0.10	20	0.87 ± 0.11	0.156

†$P < 0.1$ (tendency to significance); *$P < 0.05$; ***$P < 0.001$.

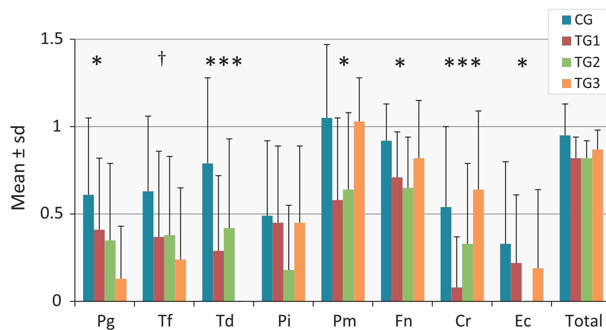

Fig. 6. Standardized bacterial contamination index (SBCI: Log_{10} (BL-CIAS)/Log_{10} (BL-PIS)) (†$P < 0.1$ (tendency to significance); *$P < 0.05$; ***$P < 0.001$).

Microbiological analysis in the present study was performed with PCR. The gold standard methodology for bacterial identification in the subgingival environment is not clear. Traditional microbiologic culturing remains the most commonly used and the reference method (Atieh 2008). However, culture techniques have shortcomings that are relevant when studying anaerobic bacteria, such as difficulties in recovering certain bacterial species and strict sampling and transport conditions.

Real-time PCR offers a faster detection time and increased sensitivity compared to traditional culture (Lau et al. 2004; Jervøe-Storm et al. 2005; Atieh 2008). This molecular biology technique is also attractive because of its ease of use and relatively low cost (Riggio et al. 1996; Eick & Pfister 2002). PCR analysis in the present study were performed by a licensed microbiologic laboratory that routinely performs culture and sensitivity tests for periodontal bacteria as recommended by Atieh (2008).

Commercial PCR microbiological assessment kits have been suggested to be adequate for detection and semi-quantitative analysis of pathogens, but not for a quantitative approach, and have also been criticized for their low sensitivity. However, the laboratory (Institut Clinident SAS) used serial dilutions of standard DNA in each reaction as external standards for absolute quantitation of the targeted pathogens. The bacterial primers used in this study are derived from previously published ribosomal 16S sequences (Slots et al. 1995; Sakamoto et al. 2001; Martin et al. 2002; Suzuki et al. 2005) and have been adapted to the real-time PCR conditions. According to the laboratory, this allowed detection limits to range between 10^2 and 10^3 for the assessed bacteria.

At the same time, the sampling process can also importantly influence the results of the microbiological analysis. Sampling can be affected at several critical steps i.e., the correct isolation of the field, the time and force of paper tips insertion in the peri-implant sulcus, the amount of sterile water inside the connection and on the abutments. Attempting to limit the influence of this sensitive step in the present investigation a single researcher carefully performed all the sampling procedures.

The patient's oral microbiological status introduces another variable which is difficult to control in a clinical investigation. The existence of teeth-implant translocation is well proven (Quirynen et al. 1999, 2000). In the present study, the microflora of healthy neighboring teeth was analyzed as control. No significant difference was observed between the groups in terms of mean bacterial loads in the gingival sulcus of teeth.

Evidence to identify the specific pathogens involved in peri-implantitis is still insufficient and reports have repeatedly indicated that peri-implant infections may be associated to a microflora different to that of chronic periodontitis (Fürst et al. 2007; Salvi et al. 2008). In the present investigation only 10 microorganisms were studied; future studies should consider more bacteria species.

Despite all the previously mentioned limitations, the present investigation represents a substantial contribution to the knowledge on microbiological leakage and sealing of implant connections. An *in vivo* clinical study after long functional loading allows a better understanding of the situation of the treated patients on daily practice than *in vitro* studies. The results obtained in the present study suggest that (1) all the connections resulted microbiologically contaminated after 5 years of functional loading, (2) the connection design might influence bacterial activity levels, especially inside the implant connection, and (3) that the effect might vary on different pathogenic species.

Further clinical studies with larger samples, analyzing more bacterial species and ideally performed at a single center should be performed to confirm conclusions drawn from the present investigation. Moreover, it remains necessary to study the clinical relevance of these differences in the microflora, especially its possible influence in the evolution of peri-implant diseases.

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