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MICROBIAL CHARACTERISTICS OF PERI-IMPLANTITIS: A CASE-CONTROL STUDY



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Submitted

ABSTRACT

Objectives

Aim of this case-control study was to compare oral microbiological characteristics of subjects with healthy peri-implant conditions and subjects with peri-implantitis while controlling for the influence of various patient-related and implant-related factors.

Material and methods

Peri-implant submucosal microbial samples were collected from 89 patients with peri-implantitis (cases) and from 71 patients with only implants with healthy peri-implant conditions (controls). Samples were analyzed using culturing techniques for presence and bacterial counts of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Parvimonas micra*, *Fusobacterium nucleatum* and *Campylobacter rectus*. Multivariable logistic regression was used to explore the association of disease status with the microbiological characteristics. The variables gender, patient age, smoking, dental status, implant function time and presence of plaque were included as potential confounders.

Results

Peri-implant disease status was significantly associated with the submucosal presence of *P. gingivalis*, *P. intermedia*, *T. forsythia*, and *F. nucleatum*. The association with disease status was most obvious for *P. intermedia* (OR 15.0, 95% CI [5.0, 44.6]) and *T. forsythia* (OR 8.6, 95% CI [3.6, 20.5]). Prevalence of *A. actinomycetemcomitans* was very low in both health (1%) and disease (3%).

Conclusions

The periodontal pathogens *P. gingivalis*, *P. intermedia*, *T. forsythia* and *F. nucleatum* seem to be associated with peri-implantitis.

INTRODUCTION

Peri-implant infection may result from a disturbance of the balance between the microbiological challenge and host response (Lindhe et al. 2008). Infection limited to the peri-implant mucosa is called peri-implant mucositis. Peri-implantitis is characterized by the additional loss of supporting bone (Zitzmann & Berglundh 2008). It has been suggested that the initiation of the disease is the result of a multi-factorial process, in which iatrogenic, mechanical, anatomical, genetic, immunological, environmental and microbiological factors can all play a role. Although it is recognized that bacteria are involved in the peri-implant disease process, they might not always be responsible for the onset of the disease (Mombelli & Décaillot 2011). It has been argued that peri-implant inflammation primarily develops as a result of an exaggerated foreign body response to a dental implant, a process which is not dependent on the presence of bacteria (Albrektsson et al. 2014). In this hypothesis, bacterial infection is a critical factor that can break the immune-tolerance towards the dental implant.

Notwithstanding the multi-factorial etiology of peri-implantitis, it is very important to understand the microbiological factors that are involved in the disease process, because this can contribute in disease prevention and assist in increasing the effectiveness of peri-implantitis treatment, that is often antimicrobial based including the use of antibiotics and antiseptics. The polymicrobial nature of peri-implant disease and the complex interactions of micro-organisms within a biofilm limit the establishment of true causative relationships between micro-organisms and peri-implant disease development/progression. However, studies have clearly shown that the submucosal biofilm in peri-implantitis differs substantially from that of implants with healthy conditions (Mombelli et al. 1987, Salcetti et al. 1997, Leonhardt et al. 1999, Hultin et al. 2002, Botero et al. 2005, Renvert et al. 2007, Shibli et al. 2008, Máximo et al. 2009, Kumar et al. 2012, Cortelli et al. 2013, Tamura et al. 2013, Da Silva et al. 2014, Persson & Renvert 2014). In order to describe the association between disease status and biofilm composition, one has to take the influence of possible biofilm-modifying factors such as age, implant function time, smoking and dental status into account (Persson & Renvert 2014, Quirynen & Van Assche 2011, De Waal et al. 2014c, De Waal et al. 2014b). However, most studies have not evaluated the role of these factors and have included only limited numbers of patients.

Therefore, the aim of the present case-control study was to compare microbiological characteristics of implants with healthy peri-implant conditions and implants with peri-implantitis while controlling for the influence of various patient-related and implant-related factors.

MATERIAL AND METHODS

The present investigation is a case-control study, comparing the presence and counts of seven putative periodontal pathogens in the submucosal peri-implant microflora of patients with peri-implantitis (cases) to the submucosal peri-implant microflora of subjects with only implants with healthy peri-implant conditions (controls). The STROBE guidelines for reporting a case-control study were followed.

Participants

Cases

Data on the peri-implantitis cases were collected from a group of patients with peri-implantitis at a private dental clinic (Quole, Center for Dental Care, Waalre, The Netherlands) from whom, as part of their regular treatment protocol, microbial samples had been collected from peri-implantitis lesions and sent for microbial analysis to the Laboratory for Oral Microbiology, University Medical Center Groningen, The Netherlands between January 2010 and April 2015. Adherence to the inclusion- and exclusion criteria was retrospectively checked from the patient files at the dental clinic by two of the authors (A.W. and Y.W.) who were unaware of the microbial outcomes of the samples. Consensus between the two authors was reached by discussion. Additional data on patient, implant and disease characteristics were collected from the patient files. Peri-implantitis was defined as presence of peri-implant inflammation (bleeding and/or suppuration on probing) combined with progressive bone loss (as judged on two consecutive radiographs) or reduced bone level (bone level ≥ 2 mm apical to the level at which the bone would 'normally' be expected). Patients who had used systemic antibiotics < 3 months prior to sample collection, implants brands other than Nobel Biocare (Nobel Biocare AG, Zürich, Switzerland) and microbiological samples that were pooled samples of both implant and teeth sites were excluded.

Controls

The control patients were prospectively and consecutively selected from the patient groups attending regular control visits at two private dental clinics (Quole, Center for Dental Care, Waalre, The Netherlands and Clinic for Periodontology Amsterdam, Amsterdam, The Netherlands) between July 2012 and March 2015.

Inclusion criteria were:

- Presence of dental implants with only healthy peri-implant conditions. A healthy peri-implant condition was defined as absence of bleeding on probing or presence of only a point bleeding on one site per implant, combined with absence of suppuration on probing, probing pocket depths ≤ 4 mm and no radiographic evidence of bone loss;
- Implant function time ≥ 5 years.

Exclusion criteria were:

- Presence of implants with peri-implant infection within the same oral cavity;
- Use of systemic antibiotics < 3 months prior to sample collection.

Informed consent was obtained from all control patients before inclusion. Additional data on patient and implant characteristics were collected during the clinical examination and from the patient files.

Variables

In both cases and controls, one microbial sample was obtained per subject. If multiple implants were present in the oral cavity a pooled sample was obtained from the implants with the deepest peri-implant pockets. Supramucosal plaque at the selected collection sites was carefully removed before sample collection. Subsequently, four sterile paperpoints were inserted in the peri-implant pockets and left in place for 10 seconds. The four paperpoints were collected (pooled) in one vial containing 1.5 ml reduced transport fluid (RTF, (Syed & Loesche 1972)) and were processed within 48 hours (Van Steenberg et al. 1993). Samples were vortexed for 30 s and tenfold serial dilutions were prepared in RTF. Aliquots of 0.1 ml were plated on 5% sheep-blood agar plates supplemented with 0.05% haemin and 0.01% vitamin K1 for isolation and growth of obligately anaerobic bacteria. Trypticase soy serumbacitracin vancomycin plates (TSBV) (Mediaproducts BV, Groningen, the Netherlands) were used for selective isolation and growth of *A. actinomycetemcomitans* (Slots 1982). Blood agar plates were incubated in 80% N₂, 10% H₂ and 10% CO₂ for up to 14 days for anaerobic growth and TSBV plates were incubated in air with 5% CO₂ for 5 days (Van Winkelhoff et al. 1985, Van Steenberg et al. 1986).

The outcome variables of the present study were presence of the periodontal pathogens *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Parvimonas micra*, *Fusobacterium nucleatum* and *Campylobacter rectus* (Zambon 1996) and mean bacterial counts (CFU/ml) and percentages of these bacterial species in culture positive subjects.

Data on the following patient and implant characteristics were collected:

- Age
- Gender
- Smoking habits (current smoker versus no/former smoker)
- Dental status (partially edentulous versus fully edentulous at the time of implant placement)
- Implant function time
- Presence of plaque (% of sampled sites with plaque)
- Presence of bleeding (% of sampled sites with bleeding)
- Probing pocket depth (mean probing pocket depth of sampled sites)

Statistical methods

Differences in demographic characteristics between cases and controls and differences in log-transformed mean bacterial counts and percentages of bacterial species between cases and controls were analyzed using Mann-Whitney *U* test for continuous variables and Fisher's exact test for dichotomous variables. A significance level (α) of 0.05 was chosen. Multiple logistic regression analysis was used to analyze potential associations of disease status (peri-implant health versus peri-implantitis), with the

outcome variables (presence/absence of seven periodontal pathogens). The variables gender, patient age, smoking habits (current versus no/former smoker), dental status (partially edentulous versus fully edentulous), implant function time and presence of plaque (% of sampled sites with plaque) were a priori identified as potential confounders. A forward selection procedure was used; confounders that significantly influenced the regression coefficient of disease status (> 10%) were step-wise included in the model until no confounders were left that exhibited a significant effect on the regression coefficient. Collinearity between the variables was checked by determining correlation coefficients ($CC > 0.70$) and variance inflation factors ($VIF > 5$). Statistical analyses were performed using IBM® SPSS® Statistics 22 (version 22.0.0.1; IBM, Armonk, NY, USA) and Graphpad Prism 5 for Windows (version 5.04; Graphpad Software, San Diego, CA, USA).

RESULTS

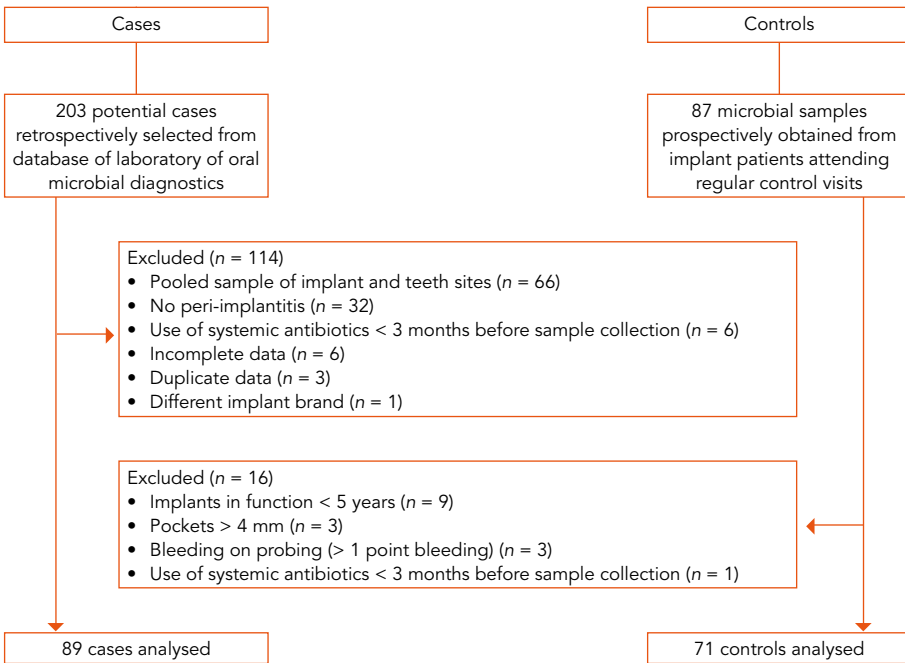
The selection process of cases and controls is depicted in Figure 1. Demographic characteristics of the included cases and controls are presented in Table 1. Significant differences existed between both groups in age, smoking habits, dental status, implant function time, mean probing pocket depth of the sampled sites and percentage of sampled sites with bleeding on probing and plaque. Detection frequencies of the periodontal pathogens in peri-implantitis (cases) and peri-implant health (controls) are depicted in Figure 2. Prevalence of *A. actinomycetemcomitans* was very low in both cases and controls (respectively 3% and 1% of the subjects). Prevalence figures of all examined periodontal pathogens were higher in cases than controls. Table 2 shows the multivariable models of the associations of disease status with the presence/absence of the periodontal pathogens after adjusting for relevant confounders.

Disease status was significantly associated with presence of *P. gingivalis* (OR 5.5, 95% CI [1.7, 17.6]), *P. intermedia* (OR 15.0, 95% CI [5.0, 44.6]), *T. forsythia* (OR 8.6, 95% CI [3.6, 20.5]) and *F. nucleatum* (OR 7.7, 95% CI [2.5, 23.9]), which means that these bacteria were significantly more often present in subjects with peri-implantitis than in subjects with healthy peri-implant conditions. *P. micra* and *C. rectus* did not show an association with disease status. The variables gender, patient age, dental status (partially edentulous versus fully edentulous), implant function time and presence of plaque (% of sampled sites with plaque) were significant confounders to the models. The log-transformed bacterial counts and mean percentages of the selected bacterial species in culture positive subjects are shown in Table 3. Total anaerobic bacterial counts and bacterial counts of *P. gingivalis*, *T. forsythia*, *P. micra*, *F. nucleatum* and *C. rectus* were significantly higher in cases than controls. No significant differences between both groups were observed in mean percentages of the selected bacterial species.

DISCUSSION

In the present study it has been shown that peri-implant disease status is significantly

Figure 1. Flow-diagram of the selection process of cases and controls.



associated with submucosal presence of *P. gingivalis*, *P. intermedia*, *T. forsythia* and *F. nucleatum*. Significantly higher detection frequencies of these pathogens were observed around implants with peri-implantitis compared to implants with healthy conditions. The association with disease status was most obvious for *P. intermedia* and *T. forsythia*, both showing high detection frequencies in peri-implantitis, but low detection frequencies in health. These two species might therefore be considered as markers for peri-implantitis.

The prevalence of *A. actinomycetemcomitans* appeared to be very low in both disease (3%) and health (1%), possibly indicating that the role of this periodontal pathogen in peri-implantitis is limited. Although peri-implantitis cases with high levels of *A. actinomycetemcomitans* have been documented (Van Winkelhoff & Wolf 2000) and although the present results seem to contradict findings from other researchers (Leonhardt et al. 1999, Hultin et al. 2002, Cortelli et al. 2013), the present findings confirm previous observations from our research group (De Waal et al. 2013, De Waal et al. 2014a).

The variables gender, patient age, dental status, implant function time and presence of plaque were significant confounders, indicating that these variables need to be taken into account when exploring the associations between peri-implant microbial characteristics and peri-implant disease status. Remarkably, smoking was no confounder, which contrasts previous observations that smoking significantly influences the

Table 1. Demographic characteristics of included subjects

Characteristics	Cases	Controls	p value
Number of patients	89	71	
Age (years; mean (SD))	61.1 (11.3)	67.7 (8.4)	$p < 0.001^*$
Gender; n subjects (%)			$p = 0.392$
male	25 (28)	25 (35)	
female	64 (72)	46 (65)	
Smoking; n subjects (%)			$p = 0.036^*$
never or former	64 (72)	61 (86)	
current	25 (28)	10 (14)	
Dental status; n subjects (%)			$p < 0.001^*$
fully edentulous at implant placement	23 (26)	37 (52)	
partially edentulous at implant placement	66 (74)	34 (48)	
Implant function time (years; mean (SD))	6.6 (3.9)	10.7 (4.2)	$p < 0.001^*$
Pocket depths of sampled sites (mm; mean (SD))	6.4 (1.7)	2.5 (0.8)	$p < 0.001^*$
% of sampled sites bleeding on probing (mean (SD))	73.4 (40.1)	5.0 (10.1)	$p < 0.001^*$
% of sampled sites with plaque (mean (SD))	30.2 (44.3)	7.5 (25.3)	$p < 0.001^*$

SD = standard deviation; * = statistically significant difference, $p < 0.05$

composition of the subgingival microbiota (Haffajee & Socransky 2001, Van Winkelhoff et al. 2001).

Unfortunately, due to lack of information it was not possible to explore the influence of the periodontal status of the remaining dentition on the microbial characteristics. This factor could have also been a confounder to the observed associations.

No differences were observed in mean percentages of the selected bacterial species in culture positive patients between cases and controls. However, the total bacterial counts of all species (except *P. intermedia*) were significantly higher in cases than controls. Similar observations have been previously made for periodontitis (Van Winkelhoff et al. 2002). The present results do not reveal whether peri-implantitis is the cause (deeper pockets contain more plaque/inflamed tissues induce more plaque formation (Socransky & Haffajee 2005)) or the result (more plaque leads to deeper pockets/disease) of the observed higher bacterial loads. Yet, it is known that the amount of plaque plays an important role in development of peri-implantitis (Heitz-Mayfield 2008). It could be hypothesized that as long as oral hygiene levels are appropriate, and consequently total bacterial loads are kept to a minimum, the influence of the mere presence and relative distribution of potentially virulent bacteria is limited. Overall, infection develops when the minimum number of bacteria required to cause infection (minimum infectious dose), is surpassed. In general, the minimum infectious dose is variable between individuals and is dependent on microbial factors, such as virulence and relative distribution of micro-organisms in a biofilm, and host-specific factors such as genetics, immunological factors, smoking and stress.

Research on peri-implant microbiology has primarily been focused on periodontal pathogens, which has been imposed by the perceived similarities between both diseases and the increased susceptibility of (treated) periodontitis patients to develop peri-implantitis (Heitz-Mayfield 2008). The development of periodontitis and peri-implanti-

Table 2. Multivariable models of association of disease status with presence of bacterial species after adjusting for relevant confounders

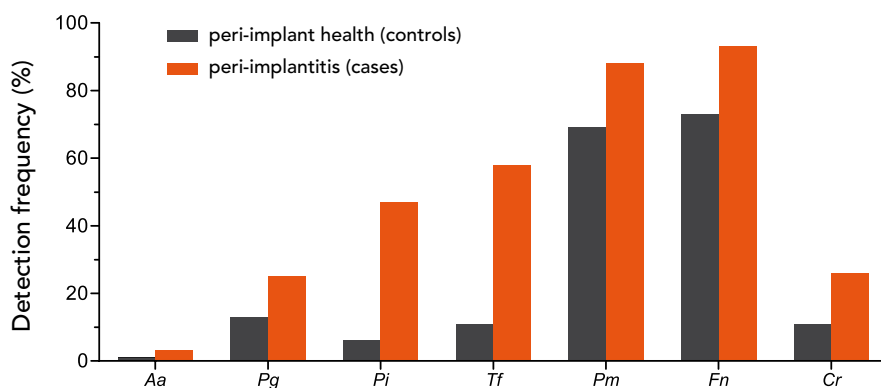
Bacterial species	Multivariable model	Multiple regression analysis		
		OR	95% CI	p value
<i>A. actinomycetemcomitans</i>	-			
<i>P. gingivalis</i>	Disease status	5.51	1.72 – 17.62	0.004*
	Implant function time	1.13	1.01 – 1.26	0.031
	Fully edentulous # / partially edentulous	4.18	1.31 – 13.36	0.016
	Age	1.07	1.02 – 1.12	0.009
	Percentage of sites with plaque	0.99	0.97 – 1.00	0.046
	Gender (female# / male)	2.43	0.86 – 6.88	0.096
<i>P. intermedia</i>	Disease status	14.97	5.03 – 44.58	<0.001*
<i>T. forsythia</i>	Disease status	8.62	3.62 – 20.52	<0.001*
	Percentage of sites with plaque	1.01	1.00 – 1.02	0.048
<i>P. micra</i>	Disease status	2.54	0.87 – 7.45	0.088
	Percentage of sites with plaque	1.03	1.00 – 1.07	0.058
	Fully edentulous # / partially edentulous	3.11	1.21 – 7.98	0.018
	Implant function time	1.06	0.95 – 1.19	0.300
	Age	1.02	0.97 – 1.06	0.466
<i>F. nucleatum</i>	Disease status	7.65	2.45 – 23.93	<0.001*
	Implant function time	1.12	0.98 – 1.27	0.099
<i>C. rectus</i>	Disease status	1.11	0.36 – 3.40	0.860
	Implant function time	0.94	0.83 – 1.07	0.344
	Percentage of sites with plaque	1.01	1.00 – 1.03	0.034
	Gender (female# / male)	0.27	0.09 – 0.86	0.027
	Age	0.98	0.94 – 1.03	0.381
	Fully edentulous # / partially edentulous	1.39	0.50 – 3.87	0.531

= reference category; OR = odds ratio; 95%CI = 95% confidence interval; * = statistically significant association ($p < 0.05$)

tis lesions clinically indeed seems to follow a similar sequence of events. However, the dynamics of the pathological processes may not be identical (Heitz-Mayfield & Lang 2010, Belibasakis et al. 2015). Recently, it has been suggested that peri-implant bone loss is not primarily the result of a bacteria-derived infection but primarily caused by an immune-modulated foreign body reaction (Albrektsson et al. 2014, Trindade et al. 2014). Secondly, a worsened inflammatory response or bacterial infection may develop as a complication, further threatening the dis-balance between implant and host (Albrektsson et al. 2014, Trindade et al. 2014). However, independent of whether a bacteria-derived infection is the primary cause of peri-implant bone loss or the secondary result of a foreign body reaction, it remains important to treat the infection, because otherwise bone loss will most definitely continue and jeopardize implant prognosis. For that reason, it remains important to understand the microbiological factors involved in the peri-implant disease process.

The observed microbial differences between peri-implantitis cases and healthy controls in the present study are in line with most other studies using culture or targeted

Figure 2. Prevalence of periodontal pathogens in peri-implant health and peri-implantitis.



Aa = *Aggregatibacter actinomycetemcomitans*; Pg = *Porphyromonas gingivalis*; Pi = *Prevotella intermedia*; Tf = *Tannerella forsythia*; Pm = *Parvimonas micra*; Fn = *Fusobacterium nucleatum*; Cr = *Campylobacter rectus*

molecular analyzing techniques such as checkerboard DNA-DNA hybridization and polymerase chain reaction, for comparison of biofilms associated with peri-implant health and disease. In general, putative periodontal pathogens such as *P. gingivalis* (Leonhardt et al. 1999, Hultin et al. 2002, Botero et al. 2005, Shibli et al. 2008, Máximo et al. 2009, Cortelli et al. 2013, Tamura et al. 2013), *P. intermedia* (Leonhardt et al. 1999, Hultin et al. 2002, Botero et al. 2005, Tamura et al. 2013), *T. forsythia* (Hultin et al. 2002, Shibli et al. 2008, Máximo et al. 2009, Cortelli et al. 2013, Persson & Renvert 2014), *P. micra* (Salcetti et al. 1997), *F. nucleatum* (Salcetti et al. 1997, Tamura et al. 2013) and *C. rectus* (Cortelli et al. 2013) are more frequently found and are found at higher levels around implants with peri-implantitis compared to implants with healthy peri-implant conditions. In addition, other species such as *Treponema denticola* (Hultin et al. 2002, Shibli et al. 2008, Cortelli et al. 2013), *Staphylococcus* spp. (Leonhardt et al. 1999, Persson & Renvert 2014), Gram-negative enteric rods (Leonhardt et al. 1999, Botero et al. 2005) and *Candida* spp. (Leonhardt et al. 1999) have also been associated with peri-implantitis. Tamura et al. (2013) suggested that asaccharolytic anaerobic gram-positive rods such as *Eubacterium* species, *Filifactor alocis* and *Slackia exigua* and gram-negative anaerobic rods, might also play an important role in peri-implantitis.

Contrastingly to the above results Renvert et al. (2007), using checkerboard DNA-DNA hybridization, found no significant differences in the microbiota at healthy implant sites in comparison to implants diagnosed with peri-implantitis. They suggested that pathogens other than those regularly studied may be involved in the peri-implant disease process. In fact, the use of new techniques, employing an open-ended, global approach for the examination of microbial communities, has indeed shown presence of previously unsuspected and unknown species (Kumar et al. 2012, Da Silva et al. 2014). However, the microbial associations that have been established for peri-implantitis based on conventional techniques, in general still hold true when new techniques are

Table 3. Log-transformed mean total anaerobic bacterial counts and mean bacterial counts of bacterial species in CFU/ml (SD) and mean percentages of bacterial species (SD) in culture positive subjects in health and peri-implantitis.

	Log-transformed bacterial counts (SD)			Mean percentages (SD)		
	Cases	Controls	p value	Cases	Controls	p value
Total anaerobic bacterial counts	8.0 (0.9)	6.7 (1.4)	< 0.001*			
<i>A. actinomycetemcomitans</i>	4.7 (0.9)	4.4		0.04 (0.03)	0.6	
<i>P. gingivalis</i>	7.1 (1.1)	5.9 (0.9)	0.003*	13.7 (15.5)	18.3 (19.0)	0.428
<i>P. intermedia</i>	6.7 (0.7)	6.1 (1.2)	0.486	5.4 (6.4)	2.5 (2.6)	0.374
<i>T. forsythia</i>	6.7 (0.8)	5.6 (1.2)	0.011*	5.6 (6.3)	2.1 (1.8)	0.105
<i>P. micra</i>	6.8 (0.9)	5.8 (1.2)	< 0.001*	11.5 (14.5)	15.5 (17.6)	0.074
<i>F. nucleatum</i>	6.7 (0.8)	5.8 (1.1)	< 0.001*	7.0 (6.6)	11.0 (11.5)	0.219
<i>C. rectus</i>	8.0 (0.9)	5.8 (1.0)	0.048*	6.4 (9.3)	4.9 (3.8)	0.642

SD = standard deviation; * = statistically significant difference ($p < 0.05$)

being applied (Kumar et al. 2012, Da Silva et al. 2014). Using 16S pyrosequencing, Kumar et al. (2012) found that both healthy and disease-associated peri-implant microbial communities demonstrated significantly lower diversity than subgingival communities, but that the microbial profile of healthy implants was significantly more diverse (showed higher species richness) than that of peri-implantitis. Using Sanger sequencing Da Silva et al. (2014) found that the biofilm associated with peri-implantitis harbored more pathogenic bacterial species from the orange complex (Socransky et al. 1998), such as *F. nucleatum*, *P. micra*, *P. intermedia* and *Campylobacter gracilis* and significantly higher percentages of clones of *Desulfobulbus*, *Dialister*, *Filifactor*, *Fusobacterium*, *Mitsuokella* and *Porphyromonas* in comparison to healthy implants.

When interpreting the results of the above mentioned studies, one should keep in mind that these studies, like the present study, are all cross-sectional. Long-term longitudinal studies with large populations of implant patients with different initial microbial profiles, which would be necessary for establishment of true causative relationships between microbiological characteristics and peri-implant disease onset/progression, have not been conducted so far. However, there is strong circumstantial evidence that several periodontal pathogens, such as *P. gingivalis*, *P. intermedia*, *T. forsythia*, and *F. nucleatum*, play a role in the development of peri-implantitis. Although new, open-ended, global, molecular analyzing techniques have revealed that the current findings do not represent a comprehensive picture of the peri-implantitis associated biofilm, they do form a basis for development of more specific (antiseptic/antibiotic) peri-implantitis treatments and measures to prevent peri-implant disease.

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