Dynamics of initial subgingival colonization of ‘pristine’ peri-implant pockets

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Key words: colonization, cross-contamination, dental plaque, implants, peri-implantitis, periodontitis, plaque growth, translocation

Abstract

Background: Periodontitis and peri-implantitis are linked to the presence of several key pathogens. The treatment of these infectious processes therefore involves the reduction/eradication of bacteria associated with periodontitis.

Methods: This prospective, split-mouth, single-blind study followed the colonization of ‘pristine’ sulci created in 42 partially edentulous patients during implant surgery (e.g. abutment connection). The hypothesis was that the composition of the maturing subgingival plaque in these ‘fresh’ peri-implant pockets would soon (within 2 weeks) be comparable to the subgingival microbiota of teeth with similar clinical parameters (reference sites), including the presence of bacteria associated with periodontitis. Per patient, four subgingival plaque samples were taken from shallow and medium pockets around implants (test sites), and teeth within the same quadrant (undisturbed microbiota as control sites), 1, 2, 4, 13, 26 and 78 weeks after abutment connection, respectively. The samples were analysed by either checkerboard DNA-DNA hybridization, or cultural techniques, or real-time polymerase chain reaction (PCR) for intra-subject comparisons (teeth vs. implant, for comparable probing depths).

Results: Checkerboard DNA-DNA hybridization and real-time PCR revealed a complex microbiota (including several pathogenic species) in the peri-implant pockets within 2 weeks after abutment connection. After 7 days, the detection frequency for most species (including the bacteria associated with periodontitis) was already nearly identical in samples from the fresh peri-implant pockets (5% and 20% of the microbiota belonging to red and orange complex, respectively) when compared with samples from the reference teeth. Afterwards (e.g. between weeks 2 and 13), the number of bacteria in peri-implant pockets only slightly increased (± 0.1 log value), with minor changes in the relative proportions of bacteria associated with periodontitis (8% and 33% of the microbiota belonging to red and orange complex, respectively). Although small differences were seen between teeth and implants at week 2 with cultural techniques, a striking similarity in subgingival microbiota was found with this technique from month 3 on, with nearly identical detection frequencies for bacteria associated with periodontitis for both abutment types.

Conclusions: This study indicates that the initial colonization of peri-implant pockets with bacteria associated with periodontitis occurs within 2 weeks.

Besides environmental factors [e.g. smoking, Bain & Moy 1993] and patient-related parameters such as bone quality (Jaffin & Berman 1991; Hutton et al. 1995), intraosseous infection (Quirynen et al. 2003) and systemic diseases or chemotherapy...
surgical trauma or bacterial contamination during implant insertion is reported to be one of the most important causes of early implant failure (for a review, see Esposito et al. 2002, 1999; Quirynen et al. 2002). Late failures are less well understood, but seem to be related to microbiological (peri-implantitis) and biomechanical (overload) challenges (for a review, see Mombelli & Lang 1998, Tonetti 1998; Mombelli 1999; Quirynen & Teughels 2003).

Peri-implantitis, an ‘inflammatory process’ affecting the tissues around an already osseointegrated implant resulting in loss of supporting bone (Albrektsson & Isidor 1994), has been associated in animal studies, as well as cross-sectional and longitudinal observations in humans, with a microbiota comparable to that of periodontitis that includes a high proportion of anaerobic Gram-negative rods, motile organisms and spirochetes. Healthy peri-implant pockets are characterized by high proportions of coccoid cells, a low ratio of anaerobic/aerobic species, a low number of Gram-negative species and low detection frequencies for bacteria associated with periodontitis (Adell et al. 1986; Lekholm et al. 1986; Bower et al. 1989; Ong et al. 1992, George et al. 1994). Implants with peri-implantitis reveal a complex microbiota that includes conventional periodontal pathogens. Species such as _Actinobacillus actinomycetemcomitans_, _Porphyromonas gingivalis_, _Tannarella forsythia_, _Prevotella intermedia_, _Peptostreptococcus micros_, _Campylobacter rectus_, _Fusobacterium_ and _Capnocytophaga_ are often isolated from failing sites (for a review, see Mombelli & Lang 1998, Mombelli 1999; Quirynen et al. 2002; Quirynen & Teughels 2003), but can also be detected around stable implants (Leonhardt et al. 2002, 2003).

In partially edentulous patients, a striking similarity in the composition of the subgingival microbiota has been observed between teeth and implants (Lekholm et al. 1986; Apse et al. 1989; Quirynen & Listgarten 1990; Koka et al. 1993, Leonhardt et al. 1993, Kohavi et al. 1994; Mombelli et al. 1995; Mengel et al. 1996; Papaiouannou et al. 1996; Gouyoubes et al. 1997; Sbordone et al. 1999; Hultin et al. 2000, 2002). Based on this similarity, it has been suggested that, at least in partially edentulous patients, teeth might act as a reservoir for the (re)colonization of the subgingival area around implants.

The dynamics of the ‘early’ subgingival colonization of the peri-implant pockets in partially edentulous patients has not received much attention in the past. In most longitudinal studies, plaque samples were taken 1 month or longer after surgery (Leonhardt et al. 1993, Mombelli et al. 1995; Mengel et al. 1996, van Winkelhoff et al. 2000), but never within the first month. This prospective, split-mouth study compared, in partially edentulous patients, the maturing microbiota in the ‘pristine’ peri-implant sulci (test sites) with the undisturbed subgingival plaque of teeth in the same quadrant [serving as control/reference sites], at several time points (week 1, 2, 4, 13, 26 and 78) using either DNA–DNA hybridization [up to week 13], or culture, or real-time polymerase chain reaction (PCR) [for the latter, only a small group of patients had been included, and only up to week 4]. Samples at longer time intervals (e.g. 26 and 78 weeks post-surgery) were included to verify the long-term stability of the subgingival microbiota. The hypothesis was that the composition of the maturing subgingival plaque in these ‘fresh’ peri-implant pockets would soon [within 2 weeks] be comparable to the subgingival microbiota of teeth in the same quadrant.

## Material and methods

### Subjects

Forty-two partially edentulous Caucasians [18 females, five smokers] volunteered for this study. They had previously at least two, 2-stage, Brånemark system® implants [Nobel Biocare®, Gothenburg, Sweden] to treat their partial edentulism in either the lower jaw [n = 17] or the upper jaw [n = 25]. All subjects were in good general health, and none of them had used antimicrobials during the 3 months prior to the study.

Most patients had a history of gingivitis or mild to moderate chronic adult periodontitis that had been treated with oral hygiene improvement (especially interdental plaque control), scaling and if necessary a root planing (n = 32 for the latter). Some patients had received periodontal surgery (n = 8), mostly (n = 6) more than 2 years prior to the study. All periodontitis therapy had been completed 3 months prior to implant insertion, thus 6 to 11 months prior to abutment connection. The number of teeth per volunteer ranged from 10 to 26 (mean 19.9), and the proportion of approximal pockets ≥ 5 mm was 7.1%, ranging from 0 to 19%. At the end of the study, only 3 patients showed several pockets ≥ 6 mm.

All participants gave their informed consent. The protocol had been approved by the Clinical Trials Committee of the University Hospital of the Catholic University Leuven.

### Experimental design

This was a prospective, split-mouth [intra-subject comparison], single-blind [microbiologists] study. Three to 8 months after fixture installation, healing abutments were inserted under local anaesthesia using either a crestal incision or a punch-out technique. The healing abutments, of commercially pure titanium, were sterile at installation and had a length of 4 to 7 mm depending on the thickness of the soft tissues. The patients were instructed to rinse twice-daily for 1 min with 0.2% chlorhexidine [Corsodyl®, SmithKline Beecham, Belgium] for 1 week. The abutments remained unloaded for the first month, or received a temporary crown/bridge. Only at the second or third month were the final abutments and the final crowns/bridges placed, so that the subgingival plaque formation during the first month remained undisturbed. At 2, 4, 13 (all subjects) and 26 and 78 (29 patients) weeks after abutment insertion, plaque samples [Table 1] were taken from implants [test sites] and teeth [reference pockets/control sites with an undisturbed plaque] within the same jaw. For the teeth, the subgingival plaque disturbance was kept to a minimum by sampling with paperpoints and pocket probing at a 6-month interval. Subgingival debridement or antibiotic therapy was not allowed. For each patient, 12 different sites were selected, three of each with the following four distinct clinical conditions: implant sites with shallow pockets (≤ 3 mm) and implant sites with deeper pockets (> 3 mm), tooth sites with shallow pockets (≤ 4 mm) and tooth sites with deeper pockets (> 4 mm). For the latter, always...
the deepest pockets within the respective jaw had been included.

For the last 19 subjects, an additional sampling at 1 week was included [Table 1] because the initial data from the first patients had shown a fast colonization of the ‘pristine’ peri-implant pockets.

**Periodontal parameters**

At each time interval except week 1, the plaque index [Silness & Løe 1964] and sulcus bleeding index [Mühlemann & Son 1971] were recorded for all sample sites [Table 1]. In order not to interfere with the initial soft-tissue healing (although the healing of the epithelial attachment after probing occurs within a week, at least in dogs, Etter et al. 2002) and early colonization [cross contamination by probing has been previously reported, Christersson et al. 1985], the peri-implant pockets were not probed during the initial months. The selection of shallow and deeper sites around implants was initially based on the thickness of the soft tissue at abutment connection. The future probing depth was estimated as the distance between bone and gingival margin minus 1.4 mm, the latter representing the average width of the soft-tissue sealing [Quirynen et al. 1991]. The teeth sites had been selected based on the probing data from the last follow-up visit some months prior to implant surgery. Pocket depth values, as well as bleeding tendency on probing (1 if present or 0 if absent, respectively), were verified at weeks 2, 26 and 78 for teeth, and weeks 26 and 78 for implants [Table 1]. The probing depth was recorded to the nearest mm by means of a Merrit B probe [Merrit B probe Hu-Friedy, Chicago, IL]. Probing was performed after plaque sampling.

**Microbiological parameters**

At each visit, samples for up to three different microbiological analyses [checkerboard DNA–DNA hybridization and/or culturing and/or real-time PCR] were taken [for details, see flow chart, Table 1]. All microbiological evaluations were performed blind. After thorough supragingival cleaning and isolation of the sites, subgingival plaque was collected using papertips [Roeko, Roeko, Langenau, Germany], with two papertips per pocket per microbiological analyses. The narrow entrance of peri-implant pockets renders plaque collection with curettes nearly impossible. The papertips were inserted for 10 s at each selected site. The first two were always used for the checkerboard analyses, the next two for culture, and the last two for real-time PCR, if applicable [i.e. when all three microbiological analyses were scheduled]. All papertips from each distinct clinical condition (peri-implant pockets ≤ 3 or > 3 mm, periodontal pockets ≤ 4 or > 4 mm, respectively) were pooled. Scaler samples are preferred for plaque samples for checkerboard DNA–DNA hybridization. In the present investigation, papertips were used instead of scalers because of concern regarding damaging tissues with recently placed implants and because of difficulties with subgingival penetration of implants when using scalers. The papertip sampling technique may recover less cells of species adherent to tooth surfaces, such as the Actinomyces, and increase the proportion of organisms adherent to the soft tissues or loosely adherent. However, since the sampling method was consistent for all sites, this does not alter the conclusions of the study.

**DNA–DNA hybridization**

Paperpoints for the checkerboard DNA–DNA hybridization analyses for the detection of levels of 40 subgingival taxa [Socransky et al. 1994] were placed in separate Eppendorf tubes containing 0.15 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) to which 0.15 ml of 0.5 M NaOH was added for fixation. The DNA–DNA hybridization was completed at The Forsyth Institute [Socransky et al. 1994]. The signals for each test species in each sample were compared with the signals from standards for each species at 10^5 and 10^6 run on each membrane [Socransky et al. 1994].

**Culture data**

Paperpoints for culture were dispersed in Reduced Transport Fluid [Syed and Loesche, 1973], homogenized by vortexing for 30 s, and processed within 24 h [for details, see Quirynen et al. 1999]. The dilutions 10^-1 to 10^-5 were plated in duplicate onto non-selective blood agar plates [Blood Agar Base II, Oxoid, Basingstoke, England], supplemented with haemin (5 mg/l), menadione (1 mg/l) and 5% sterile horse blood. After 7 days of anaerobic (80% N₂, 10% CO₂ and 10% H₂) and aerobic incubation at 37°C, respectively, the total number of colony forming units (CFU/ml) was counted. For each pigmented [black, green, brown] colony ‘type’ on the representative anaerobic plate, every third colony was subcultured and identified. From the original anaerobic blood agar plates, the number of CFU/ml of P. micros was also estimated based on its typical colony morphology. The dilutions 10^-1 to 10^-5 were also plated on 3 selective media: a Hammond medium for the detection of C. rectus [Hammond 1988], a CVE medium for the detection of Fusobacterium nucleatum [Walker et al. 1979] and a highly selective medium for the detection of A. actinomycetemcomitans [Alsina et al. 2001].

**Real-time PCR**

Once the commercial Perio Diagnostics® test [Meridol®, Gaba Dental, Münchenstein, Switzerland] became available in the soft tissues, samples were also collected [from last nine patients] for this real-time PCR test, identifying the presence of A. actinomycetemcomitans, P. gingivalis,

### Table 1. Flow chart of the study with timing for samples for each microbiological technique and timing of registration of periodontal parameters (within brackets, the number of patients selected for these analyses)

<table>
<thead>
<tr>
<th>Timing</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 4</th>
<th>Week 13</th>
<th>Week 26</th>
<th>Week 78</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>For checkerboard</td>
<td>(Last 19)</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>-</td>
</tr>
<tr>
<td>For culturing</td>
<td>-</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>For PCR</td>
<td>(Last 9)</td>
<td>(Last 9)</td>
<td>(Last 9)</td>
<td>(Last 29)</td>
<td>(Last 29)</td>
<td>-</td>
</tr>
<tr>
<td>Periodontal parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plaque index</td>
<td>-</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Gingivitis index</td>
<td>-</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bleeding on probing</td>
<td>-</td>
<td>x (t)</td>
<td>x (t)</td>
<td>x (t)</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Probing depth</td>
<td>-</td>
<td>x (t)</td>
<td>x (t)</td>
<td>x (t)</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction; x (t) = teeth only.
Quirynen et al. Early colonization implants

T. forsythia, Treponema denticola, F. nucleatum, and P. intermedia. This new technique uses, in addition to primers specific for the respective pathogen DNA/RNA, another species-specific DNA fragment [TaqMan probe] in the same PCR reaction. This fragment binds within the target sequence. While the target sequence is amplified, the exonuclease activity of the Taq polymerase cleaves the fluorescently labelled TaqMan probe from the target sequence and deletes it. This degradation of the probe releases a fluorescent signal, which can be monitored online in the reaction by automatic laser detection. The intensity of the fluorescent signal thus represents the amount of product formed and is directly proportional to the initial amount of the pathogen in the patient sample [Jerve-Storn et al. 2005].

For an intra-subject comparison of the subgingival microbiota between implant and teeth, over a certain period, patients who missed an appointment and/or for whom samples had not been taken [due to the intake of antibiotics in the 3 months prior to sampling] were excluded. The number of patients with data for the specific appointments were as follows: 19 for observations at week 1 and 2; 33 for observations at weeks 2, 4 and 13; 39 for observations at weeks 2 and 13; and 29 for weeks 2, 4, 13, 26 and 78. Only for nine patients, samples for real time PCR data were collected. For each experimental period discussed in this paper [e.g. week 2 vs. week 4 vs. week 12], only those patients who were available at each of these specific appointments were considered, in order to allow a longitudinal evaluation.

The significance of differences among the four site types [clinical conditions] in the counts [DNA–DNA hybridization] of the 40 test species (at weeks 2, 4 and 13, or weeks 1 and 2, respectively) as well as in the total amount of DNA count and/or of the number of CFU [aerobe or anaerobe] was sought using the Kruskal–Wallis test, with adjustment for multiple comparisons [Socransky et al. 1991]. Because of differences in probing depth for the moderate pockets [teeth vs. implants], an additional analysis with only the shallow pockets had been included. For the real-time PCR and for detection frequencies, only descriptive analyses have been included.

In order to quantify numerically the evolution in microbial load within the peri-implant pocket in comparison with the undisturbed plaque from teeth, the data of key pathogens at shallow pockets [teeth and implants] were selected for regression analysis.

Results

Periodontal clinical parameters

The mean periodontal parameters for the 29 patients who completed the entire follow-up period did not differ significantly ($P>0.1$) when shallow pockets around teeth and implants were compared with each other [Table 2]. For the medium pockets, however, the scores were often significantly higher around teeth ($P \leq 0.005$), especially the plaque index and the bleeding on probing scores. The probing depth for the deeper sites unfortunately was also slightly higher for the teeth than for the implants.

Most parameters remained unchanged over time ($P>0.10$). Between shallow and medium pockets, within tooth sites, and to a lower extent within implant sites, higher scores were often recorded for the deeper sites, especially concerning bleeding on probing, plaque index and probing depth ($P \leq 0.005$); the latter can of course be

### Table 2. The different periodontal parameters (rows) for different conditions (columns: implant with shallow or deeper pocket, and tooth with shallow or deeper pocket, respectively) as observed at weeks 2, 13, 26 and 78, for only those patients that were present at each of these four appointments (n = 29)

<table>
<thead>
<tr>
<th>Week</th>
<th>Implant ≤ 3 mm</th>
<th>Implant &gt; 3 mm</th>
<th>Tooth ≤ 4 mm</th>
<th>Tooth &gt; 4 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Plaque index</td>
<td>2</td>
<td>0.6</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Gingivitis index</td>
<td>13</td>
<td>0.6</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Bleeding on probing</td>
<td>26</td>
<td>0.6</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Probing depth (mm)</td>
<td>78</td>
<td>0.4</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>p 1 = probability, if significant, for difference between implants with shallow pockets vs. medium pockets, p 2 for teeth with shallow vs. medium pockets, p 3 between implants and teeth with shallow pockets and p 4 between implants and teeth with medium pockets, respectively. N, not significant.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
explained by the cut-off values during the selection of the different sites.

**Microbiological observations: long term**

Checkerboard: colonization during the first 3 months: week 2 vs. week 4 vs. week 13 (33 patients)

Figure 1 shows the detection frequencies for members of the red and orange complexes under the different clinical conditions (Socransky et al. 1998) at 2, 4 and 13 weeks. The detection frequencies of these species in samples from peri-implant pockets ($\leq 3$ or $>3$ mm) were similar to those observed in samples from the shallow periodontal sulci of natural teeth. The difference in detection frequency in peri-implant pockets ($\leq 3$ or $>3$ mm) vs. shallow periodontal pockets for the 16 species in this figure was $\leq 3$ in 78 out of the 96 observations [mean difference in detection frequency $= 1.5$]. For moderate pockets around teeth, the detection frequencies of these bacteria were higher (mean difference in detection frequency $= 6.2$). Over the 13 weeks of observation, only minor changes in detection frequencies could be observed.

The total DNA probe counts (for the 40 taxa together) in samples from weeks 2, 4 and 13 are shown in Table 3. At week 2, the total DNA probe counts for peri-implant pockets were negligible [increase $\pm 0.1$ log].

The changes in the mean DNA probe counts for the 40 test species colonizing the peri-implant pockets and teeth over time are shown in Fig. 2. The data for the teeth changed little over time, with the moderate pockets harbouring more members of the red and orange complexes than the shallow pockets ($P < 0.005$). The changes over time in the counts for peri-implant pockets were negligible [increase $\pm 0.1$ log].

The changes in the mean DNA probe counts for the 40 taxa together in samples from weeks 2, 4 and 13 are shown in Table 3. The differences between implants and teeth were small, irrespective of probing depth and time point [all data within $0.3$ log, statistically different for week 2 only with $P = 0.003$]. The changes over time in the counts for peri-implant pockets were negligible [increase $\pm 0.1$ log].

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![Fig. 1](image-url) Detection frequency for members of the red and orange complex and A. actinomycetemcomitans in samples ($n = 33$) from implants [I] with shallow and medium pockets, respectively, taken at 2, 4 and 13 weeks post-abutment connection. The mean detection frequency, averaged over all three visits, for samples of teeth (T) with shallow and moderate pockets and an undisturbed plaque is included for comparison. The y-axis indicates the number of sampled sites positive for each species. Abbreviations: C. gracilis (C.g.), C. rectus (C.r.), C. showae (C.s.), E. nodatum (E.n.), F. nucleatum ss nucleatum (F.n.n.), F. nucleatum ss polymorphum (F.p.n.), F. nucleatum ss vincentii (F.n.v.), F. periodonticum (F.p.), P. micros (P.m.), P. intermedia (P.i.), P. nigescens (P.n.), S. constellatus (S.c.), T. forsythia (T.f.), P. gingivalis (P.g.), T. denticola (T.d.) and A. actinomycetemcomitans (A.a.).

**Table 3. Relative proportion for each complex (based on groupings suggested by Socransky et al. 1998) in relation to total amount of examined plaque (total in log value)**

<table>
<thead>
<tr>
<th>Condition (mm)</th>
<th>Week</th>
<th>Total ($\times 10^6$)</th>
<th>Relative proportions in % per complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Red</td>
</tr>
<tr>
<td>I $\leq 3$</td>
<td>2</td>
<td>1.1</td>
<td>7.2</td>
</tr>
<tr>
<td>I $\leq 3$</td>
<td>3</td>
<td>4.1</td>
<td>5.6</td>
</tr>
<tr>
<td>I $\leq 3$</td>
<td>13</td>
<td>12.2</td>
<td>7.8</td>
</tr>
<tr>
<td>I $\leq 3$</td>
<td>4</td>
<td>2.1</td>
<td>7</td>
</tr>
<tr>
<td>I $\leq 3$</td>
<td>13</td>
<td>1.9</td>
<td>8.8</td>
</tr>
<tr>
<td>I $\leq 3$</td>
<td>4</td>
<td>2.5</td>
<td>6.5</td>
</tr>
<tr>
<td>I $\leq 3$</td>
<td>13</td>
<td>1.9</td>
<td>8.8</td>
</tr>
<tr>
<td>T $\leq 4$</td>
<td>2</td>
<td>2</td>
<td>10.5</td>
</tr>
<tr>
<td>T $\leq 4$</td>
<td>4</td>
<td>2.3</td>
<td>12.1</td>
</tr>
<tr>
<td>T $\leq 4$</td>
<td>13</td>
<td>2.1</td>
<td>11.6</td>
</tr>
<tr>
<td>T $\leq 4$</td>
<td>4</td>
<td>3.7</td>
<td>19.7</td>
</tr>
<tr>
<td>T $\leq 4$</td>
<td>4</td>
<td>3.8</td>
<td>13.2</td>
</tr>
<tr>
<td>T $\leq 4$</td>
<td>13</td>
<td>3.5</td>
<td>14.9</td>
</tr>
</tbody>
</table>

Actin, actinomyces; Data are grouped per conditions (implant with shallow or deeper pocket, and tooth with shallow or deeper pocket, respectively) and per week (weeks 2, 4 and 13) for patients that did not miss any appointment ($n = 33$).
Counts×10^5

A. gerencseriae
A. naeslundii 1
A. naeslundii 2
A. odontolyticus
V. parvula
S. gordonii
S. intermedius
S. mitis
S. oralis
S. sanguis
A. actinomyceseomcomitans
C. gingivalis
C. ochracea
C. spuriipena
E. corrodens
C. gracilis
C. rectus
C. showae
E. nodatum
F. nucleatum ss nucleatum
F. nucleatum ss polymorphum
F. nucleatum ss vincentii
P. periodonticum
P. micros
P. intermedia
P. nigrescens
S. constelatus
T. forsythia
P. gingivalis
T. denticola
E. saburreum
G. morbillorum
L. buccalis
N. mucosa
P. acnes
P. melaninogenica
S. anginosus
S. noxia
T. socranski

Fig. 2. Microbial profiles of the mean counts [×10^5] of 40 microbial taxa in subgingival plaque samples taken from the four sample types (teeth or implants, shallow or moderate pockets) at 2, 4 and 13 weeks after abutment connection (n = 3). The profiles represent the mean counts derived by averaging the counts of each species across subjects for each site type and each time point. The species have been ordered according to the complexes described by Socransky et al. (1998). Significance of differences among sample types was tested using the Kruskal–Wallis test and adjusted for multiple comparisons (Socransky et al. 1991; \*P<0.05, \**P<0.01, ***P<0.001.

Counts of ≥ 10^5 for members of the red and orange complexes remained stable around the teeth [22% and 15% for shallow pockets, 40% and 27% for moderate pockets, respectively], but showed a clear increase with time for the samples from implants [3% vs. 12% vs. 15%, and 4% vs. 9% vs. 15% for shallow pockets; 8% vs. 12% vs. 21%, and 11% vs. 12% vs. 13% for moderate pockets, at weeks 2, 4, and 13 for red and orange complex species, respectively].

The number of key pathogens in the shallow pockets around teeth and implants was compared with each other per time interval, using regression analyses (Fig. 3). At weeks 2 and 4, the correlation was weak (r^2 = 0.02, 0.04). At week 13, however, the number of key pathogens around implants approached the value for teeth [implants harbour 50% of plaque amount around teeth, r^2 = 0.36].

Checkerboard: colonization during the first half year (24 patients)

After 26 weeks, the total DNA probe count was nearly identical (P = 0.20) for all conditions (1.6 × 10^6, 1.9 × 10^6, 2.6 × 10^6, 3.3 × 10^6 for implants and teeth with shallow and moderate pockets, respectively). The mean DNA probe counts for the 40 test species colonizing the peri-implant pockets and teeth at weeks 13 and 26 are shown in Fig. 4. The data for teeth remain similar over time, with the moderate pockets harbouring more members of the red and orange complex than the shallow pockets, although the differences did not reach a level of significance. The composition of the subgingival plaque from shallow and moderate peri-implant pockets is comparable, and quite similar to the microbiota from the shallow pockets around teeth (with the exception of P. nigrescens).

Bacterial culture data

The number of bacteria cultured under aerobic or anaerobic conditions in samples from peri-implant pockets (Table 4) showed only a minor increase (<0.3 log), between week 2 and week 13 (P>0.05). Afterwards, the changes were negligible. The difference between peri-implant pockets with shallow and medium probing depths was minimal, with the latter harbouring ±0.2 log more bacteria, except for week 2, where the difference was slightly higher (P<0.01). In general, the microbial load around the implants corresponded well to that around teeth with shallow pockets, but was always lower than the load around teeth with deeper pockets. The ratio of anaerobic/aerobic species was relatively constant (≤3) for both types of peri-implant pockets as well as for teeth with shallow pockets. For teeth with deeper pockets, this value was slightly higher (≤6).

The detection frequency for key pathogens by culture is also highlighted in Table 4. At week 2, the implants presented with clearly lower detection frequencies (about 50% less) when compared with the teeth. Three months after abutment connection, 2/29 harboured P. gingivalis and/or Actinomyceseomcomitans, 16/29 P. intermedia and 17/29 C. rectus, scores that were comparable to those obtained for teeth with shallow pockets. 18 months after abutment connection, these values...
Further increased to 8/29, 20/29 and 20/29 for *P. gingivalis*, *C. rectus*, and *P. intermedia*, respectively. At this time point, the detection frequencies reached the levels obtained for teeth with moderate pockets.

**Microbiological observations: short-term**

Checkerboard: early colonization: week 1 vs. week 2 (19 patients)

After 1 week of colonization including the use of a chlorhexidine mouthrinse, nearly $10^6$ DNA probe counts were found at the ‘initially pristine’ peri-implant pockets. This number was lower ($P=0.02$) than that detected for teeth ($1.4 \times 10^6$ for shallow pockets, $2.9 \times 10^6$ for moderate pockets). However, at week 2, this difference disappeared (<0.2 log, $P=0.13$, $1 \times 10^6$ for shallow pockets, $1.2 \times 10^6$ for moderate peri-implant pockets).

At weeks 1 and 2, the ‘pristine’ peri-implant pockets already harboured members of the red ($\pm 5\%$) and orange ($\pm 20\%$) complexes. Counts $\geq 10^5$ for members of the red and orange complexes were more frequently observed around teeth [15\% and 12\% for shallow pockets, 40\% and 23\% for moderate pockets, respectively], but remained relatively scarce for the samples from implants [0–0\% and 0–2\% for shallow pockets, 2–2\% and 4–5\% for moderate pockets at weeks 1–2, for the red and orange complex, respectively].

The mean DNA probe counts at weeks 1 and 2 for the 40 specific species are shown in Fig. 5. The profiles were very similar for the shallow and deeper peri-implant pockets, and between weeks 1 and 2 only minor changes occurred. The red and orange complex species exhibited higher mean counts in samples from teeth, particularly those with >4 mm pockets, when compared with implants. After adjusting for multiple comparisons, significant differences among sample locations were observed for several members of the red and orange complex (*Campylobacter gracilis*, *Fusobacterium periodonticum*, *F. nucleatum*, *P. micros*, *Prevotella nigrescens*, *T. forsythia* and *P. gingivalis*), especially at week 1.

**Discussion**

The present investigation demonstrated that in the presence of teeth [partial edentulism], a complex subgingival microbiota is established in a ‘pristine’ peri-implant pocket within 1 week. Indeed, after 7 days of colonization (even while using chlorhexidine mouthrinse), both the DNA–DNA hybridization and the PCR technique revealed high proportions of key pathogens in the fresh peri-implant pockets. This microbiota appeared at peri-implant sites whether they were of shallow or medium depth. From week 2 onwards, only minor increases in total numbers of species were observed, with a nearly unchanged proportional composition, except for members of the red and orange complexes, for whom a further increase up to week 13 could be observed. At week 13, the peri-implant pockets harboured nearly 50\% the amount of key pathogens (in numbers) observed around the teeth. In a study similar to this one [Van Winkelhoff et al. 2000], but with samples taken 1, 7 and 13 months after abutment connection, it also took up to month 7 before a relative stable subgingival microbiota was detected. These authors, however, only used culture techniques, so that their observations are basically in agreement with our findings.

The exact mechanism by which these peri-implant pockets are colonized is still unclear. Bacteria may grow down from the supragingival area, although subjects were using a chlorhexidine mouthrinse. Bacteria may directly infect the blood cloth formed after the placement of the abutment, the

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**Fig. 3.** Regression analyses for correlation between number of key pathogens (*Actinomyces actinomyces*, *Peptostreptococcus micros*, *Prevotella intermedia*, *Tannerella forsythia*, *Porphyromonas gingivalis* and *Treponema denticola*) in shallow pockets around implants and teeth, per time interval [a, week 2; b, week 4 and c, week 13, including the data of 33 patients].
Fig. 4. Microbial profiles of the mean counts ($ \times 10^5$) of 40 microbial taxa in subgingival plaque samples taken from the four sample types (teeth or implants, shallow or moderate pockets) at 13 and 26 weeks after abutment connection ($n = 24$). The profiles represent the mean counts derived by averaging the counts of each species across subjects for each site type and each time point. The species have been ordered according to the complexes described by Socransky et al. (1998). The significance of differences among sample types was tested using the Kruskal–Wallis test and adjusted for multiple comparisons (Socransky et al. 1991); *P<0.05, **P<0.01, ***P<0.001.
dontitis were detected at peri-implantitis free implants via checkerboard DNA–DNA hybridization (Hultin et al. 2002; Quirynen et al. 2005), although in low numbers. Lee and et al. (1999a, 1999b) even found periodontal pathogens, although infrequently, in the first year of loading.

The high detection frequencies for bacteria associated with periodontitis in this study may be explained by the microbiological techniques used. Several papers have compared the detection sensitivity and specificity of Checkerboard DNA–DNA hybridization with cultural techniques and PCR (Papapanou et al. 1997; Sunde et al. 2000; Siqueira et al. 2001, 2002; Watson et al. 2004). In general, these studies reported a clearly higher detection frequency for *P. gingivalis* and *A. actinomycetemcomitans* when using the two molecular tests, compared with cultural techniques, with only small differences between the former. When (real-time) PCR techniques were compared with conventional cultural techniques, also higher detection sensitivity and specificity were obtained (Riggio et al. 1996; Boutage et al. 2003). Leonhardt et al. (2003) compared cultural techniques with checkerboard DNA–DNA hybridization for samples from Brånemark implants. They reported clearly higher detection frequencies for the latter, even when a high cut-off point (e.g. $>10^6$) was used. In our study, a significant number of peri-implant sites were positive even when the cut-off point was $>10^5$, but a $10^6$ level was rarely observed. Even for teeth with moderate pockets, a $10^6$ level was infrequently reached ($<5\%$ of samples for specific species). While cultural techniques may be considered by some as the gold standard, many reports suggest that the newer microbiological techniques may improve the predictability and accuracy of microbiological test in relation to disease. Another possibility is that false positives may have occurred due to contamination during sample handling.

### Table 4. Culture data (number of colony forming units per ml, as well as detection frequency for specific species) for different conditions (implant with shallow or deeper pocket, and tooth with shallow or deeper pocket, respectively), observed at weeks 2 and 13, and 2, 13, 26 and 78, for patients that did not miss any appointment ($n = 39$ and 29, respectively)

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CFU, colony forming units.
to binding of DNA probe or antibody conjugate to protein or other substances in large plaque samples. This is unlikely when small samples are analysed (Socransky et al. 2004) as in the present investigation.

Comparison between implants with medium and shallow pockets showed similar proportions of red and orange complexes (58% and 45%, respectively). However, deeper pockets (often related to the trimming during abutment insertion and in this group of patients not pointing to peri-implantitis) harboured more pathogens. This is in agreement with other studies, even though the difference between deep and shallow pockets in this study was small. Papaioannou et al. (1995) reported that the probing depth was closely related to the pathogenicity of the microbiota (the deeper the pocket, the higher the proportion of spirochetes and motile organisms). George et al. (1994) reported a positive correlation between probing pocket depth and/or intra-oral

Fig. 5. Microbial profiles of the mean counts ($10^5$) of 40 microbial taxa in subgingival plaque samples taken from the four sample types [teeth or implants, shallow or moderate pockets] at 1 and 2 weeks after abutment connection ($n = 19$). The profiles represent the mean counts derived by averaging the counts of each species across subjects for each site type and each time point. The species have been ordered according to the complexes described by Socransky et al. (1998). The significance of differences among sample types was tested using the Kruskal–Wallis test and adjusted for multiple comparisons (Socransky et al. 1991); $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Fig. 6. Detection frequency for members of key pathogens in samples ($n = 9$) from implants (I) with shallow and moderate pockets, respectively, taken at 1, 2 and 4 weeks post-abutment connection. The mean detection frequency, averaged over all three visits, for samples of teeth (T) with shallow and moderate pockets and an undisturbed plaque is included for comparison. The y-axis indicates the number of sampled sites positive for each species. Abbreviations: F. nucleatum (F.n.), P. intermedia (P.i.), T. forsythia (T.f.), P. gingivalis (P.g.), T. denticola (T.d.) and A. actinomycetemcomitans (A.a.).

Quirynen et al. Early colonization implants

exposure time, and the presence of *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans*.

The presence of key pathogens does not necessarily jeopardize the future of the implants. Hultin et al. (2002) concluded that the relative amount and not the presence of these pathogens was linked with peri-implantitis. Indeed, none of the healthy implant sites reached a ≥10^6 threshold level for individual key pathogens in contrast to sites with peri-implantitis (35%–88% of sites positive for different target species). Similar observations were made by Leonhardt et al. (2002), who followed their partially edentulous patients, rehabilitated with Branemark implants, for a period of up to 10 years. They also could not see a difference in clinical outcome between implants positive or negative for key bacteria associated with periodontitis.

In the present investigation, the observation of colonization of a sterile hard surface placed subgingivally by a complex microbiota within days was unexpected. Whether this related to the special environment offered by the blood coagulum, a situation similar to that after mechanical debridement of periodontal pockets, needs further investigation.

**Conclusion**

The present study indicated that bacteria associated with periodontitis could colonize peri-implant pockets within a week. Their numbers were relatively low initially, but appeared to reach a stable level after 3 months.

**Acknowledgements:** This study was supported by a grant of the ITI Research foundation (Switzerland), a grant from the Catholic University Leuven (OT/03/52) and a research grant DE14368 from the National Institute of Dental and Craniofacial Research, USA. Daniel van Steenberghe is holder of the P-I Braånmark Chair in Osseointegration.

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