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ORIGINAL ARTICLE

Influence of bacterial colonization of the healing screws on peri-implant tissue

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Abstract *Background/purpose:* A bacterial adhesion to implant surfaces is a first stage of peri-implant mucositis and peri-implantitis. The aim of this longitudinal study was to examine the quantitative and qualitative biofilm formation on healing screws and the presence of periodontal pathogens in peri-implant crevicular fluid (PCF). In each of the 30 participants, one healing screw was adapted to every single implant.
Materials and methods: Thirty patients, 18 females and 12 males, were selected. Thirty healing abutments were left *in situ* for 20, 30 and 90 days. At regular times, the presence/absence of bleeding on probing (BOP) was determined. The specific periodontal pathogens were determined in PCF, by polymerase chain reaction (PCR). After 20, 30 and 90 days, the healing screws were removed and analyzed to establish the total bacterial count by a culture method.
Results: BOP+ increased significantly after 90 days. Biofilm was detected on all healing abutments and the number of cultivable oral flora showed a significant increase from 20 days to 30 days and to 90 days. At 90 days, the anaerobic counts constituted the bulk of plaque examined and *Veillonella* spp. were present in higher percentages levels (19.82%) among the Gram-negative bacteria. No significant differences among the experimental groups were detected in the frequencies of detection of each monitored bacteria in PCF.

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Conclusion: The healing screws left *in situ* for a period of 90 days caused a peri-implant inflammation and the presence of periodontal pathogenic bacteria in the peri-implant sulcus, due to the plaque accumulation on screw surfaces.

Introduction

The use of osseointegrated oral implants has become quite popular in recent years, for replacement of missing teeth. The inflammatory reactions of the tissue around the implants and the formation of a reservoir for pathogens, sometimes leading to implant failure, could be caused by different factors as, for example, plaque accumulation on implant surfaces. Bacterial adhesion to implant surfaces is a first stage of peri-implant mucositis and peri-implantitis, and a positive correlation has been found between oral hygiene and marginal bone loss around implants in the edentulous mandible.[1,2] Biofilm accumulates on all surfaces in the mouth, regardless as to whether these are natural, artificial or dental materials, but many studies suggest that the composition of supragingival bacterial community around healthy teeth is similar to that found around implants.[3]

While a considerable amount has been reported about bacterial adhesion to enamel, relatively little information is available about the mechanisms of bacterial interactions with implant materials in the oral cavity.

Surface properties of transgingival implant components are important determinants in bacterial adhesion, and factors other than the surface roughness play a role in the adsorption of proteins and the adhesion of bacteria to the implant components; in particular, the type of bacterium, the concentration, the growth phase, the mechanical retention, the physiochemical characteristics of the materials, hydrophobicity, zeta potential, and surface free-energy all affect colonization to varying degrees.[4–6]

Several studies have demonstrated that both the quality and the quantity of plaque adhesion on the implant surface are important for the long-term success of dental implants.[4,7]

The aim of this controlled longitudinal study was to examine the quantitative and qualitative biofilm formation on healing abutments left *in situ* for 20, 30 and 90 days. Also, this study proposed to evaluate whether periodontal pathogens were present in peri-implant crevicular fluid (PCF) around healing abutments.

Materials and methods

Patient selection and study design

Thirty patients, 18 females and 12 males, aged between 40 and 55 years (mean age = 46.7 years) and without important pathologies, participated in the study. Consent was given by signing a protocol (Privacy Law DL 196/2003).

The participants were selected on the basis of good periodontal health and no signs of mouth breathing. One week prior to the beginning of the study, supragingival plaque was professionally removed, oral hygiene procedures were established and ideal gingival health conditions were obtained in all volunteers (Loe and Silness gingival index = 0) and none of the subjects chosen for the study had used mouthrinses or had taken antibiotics during the previous 6 months. The radiographical and surgical evaluation of the bone density at the moment of the insertion was, for all implants (Astra Tech, DENTSPLY Friadent, Mannheim, Germany), D2 (thick cortical bone with compact and dense internal trabeculation) to D3 (cortical bone with less thickness and less dense cancellous bone) quality for all implants. All of these implants were submerged, and a healing screw was positioned on all implants after 3 months in the mandible and 6 months in the maxilla. In each of the 30 participants, one healing screw was adapted to every single implant.

Neither cleaning procedures, nor agents for chemical plaque control, were applied to the healing screw for the duration of the test period. The healing abutments were left *in situ* for the determined times of 20, 30 and 90 days. At previously established times (20, 30 and 90 days), the Full Mouth Plaque Score (FMPS) and Full Mouth Bleeding Score (FMBS) were recorded in the natural dentition of the patients. On each dental implant site, the presence or absence of bleeding on probing (BOP) within 15 seconds after probing with a 20 g controlled-force probe, were determined at every recall visit, in four sites (buccal, oral, mesial and distal) around the fixture.

Before clinical examination, microbiological analysis was performed.

Prior to PCF collection, all supragingival plaque, if present, was removed with cotton pellets; the sites selected for PCF sampling were then isolated with cotton rolls and PCF was collected by the insertion of a #40 standardized sterile endodontic paper point into the base of the sulci, until slight resistance was perceived. The paper points were left *in situ* for 30 seconds, and were transferred to plastic vials and immediately sent to the microbiological laboratory for determining specific periodontal pathogens, by polymerase chain reaction.

After 20, 30 and 90 days, the healing screws were removed and gently placed inside sterile plastic vials containing 1 mL of reduced transport fluid (RTF), and immediately sent to the microbiological laboratory for determination of the total bacterial count by the culture method. Later, new screws were placed.

Microbiological procedures

Culture conditions

Biofilm formation on healing abutments was analyzed quantitatively and qualitatively by bacterial culturing. All

samples were incubated in 0.25% trypsin for 45 minutes, in a shaking water bath at 37°C. Following the trypsin step, the contents of the tube were vortexed thoroughly and subjected to a series (five series) of 10-fold dilutions in 0.1 M phosphate buffer. Aliquots of 100 μL from undiluted suspension and each diluted were spread in duplicate onto Columbia blood agar (CBA) plates (Oxoid Italia SpA, Garbagnate Milanese, Milan, Italy) and trypticase soy agar plates supplemented with 5% defibrinated sheep blood (ETSA), to quantify the number of all cultivable oral bacteria, which was recorded as the colony count that formed per mL (CFUs/mL) on the growth plate. In particular, the CBA plates were used to cultivate anaerobic bacteria under strictly anaerobic conditions at 37°C for 7–12 days in an anaerobic chamber (80/10/10, N2/H2/CO2; Don Whitley Scientific Ltd; International PBI SpA, West Yorkshire, UK) and the ETSA plates were incubated aerobically at 37°C for 24–48 hours. Isolation of microorganisms was pursued by methods previously reported.[8] In particular, for some bacterial strains, special microbiological procedures were applied. The samples were also plated onto Mitis Salivarius Agar (MSA) (Difco, Becton, Dickinson and Company, Sparks, MD, USA) containing 1% Chapman tellurite solution, to enumerate oral streptococci and enterococci; onto MRS Agar (Difco, BD) to assess *Lactobacillus* spp.; onto brain heart infusion agar (BHNM) enriched with nalidixic acid (30 mg/L) (Sigma Aldrich, Milan, Italy) and metronidazole (10 mg/L) (Sigma Aldrich) to assess *Actinomyces* spp.; and trypticase soy crystal violet erythromycin (4 mg/L) (CVE), to assess *Fusobacterium* spp.

The purification and characterization of clinical isolates were lead as described by a previous study.[9] Finally, a definitive identification of all representative isolates was then obtained by subculturing onto Brucella blood agar (Oxoid), followed by inoculation of purified cultures onto a commercially packaged automated system (bioMérieux Italia SpA, Marcy-l'Etoile, France). For each microbial species, data were recorded as the count of CFUs/mL on the growth plate. The total bacterial counts, obtained by adding data of the count of CFUs/mL on the CBA plate and ETSA plate, and the frequencies of detection for each microbiota, were also reported.

DNA-extraction

The PCF was analyzed by polymerase chain reaction. The nucleic acids were extracted within 24 to 48 hours of specimen collection. The samples were vortex-mixed and centrifuged to collect the cells. The pellets were resuspended in 300 μL lysis buffer (50 mM Tris, 10 mM EDTA and 10% SDS, pH 7.4) plus lysozyme (5 mg/mL), and incubated at 37°C for 1 hour. Proteinase-K was added, and after 1 hour incubation at 65°C, the DNA was extracted with phenol and chloroform-isoamyl alcohol treatment. The nucleic acids were precipitated in ethanol, washed with 70% (v/v) ethanol, and resuspended in sterile water. The DNA extracted from each sample was assayed by multiplex polymerase chain reaction (PCR), for the detection of *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), *Campylobacter rectus* (*C. rectus*), *Eikenella corrodens* (*E. corrodens*), *Fusobacterium nucleatum* (*F. nucleatum*), *Prevotella intermedia* (*Prev. intermedia*), *Porphyromonas gingivalis* (*Porph. gingivalis*), *Tannerella forsythia* (*Tann. forsythia*) and *Treponema denticola* (*Trep. denticola*).[10]

PCR-detection

The multiplex PCR was performed using specific primers for the 16S rRNA gene of each bacterium. The PCR amplification reactions were carried out in a 100 μL final volume of the reaction mixture that included 10 μL of the DNA sample. The remaining 90 μL of the reaction mixture contained 30 pmol of each primer, 200 μmol of a mixture of deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, PCR buffer (10 mM Tris-HCl, pH 8.0), 50 mM KCl, and 2.5 U Hot Start Taq DNA Polymerase. The PCR protocol was as follow: 98°C for 15 minutes, followed by 40 cycles at 95°C for 30 seconds, 1 cycle at 60°C for 1 minute, 1 cycle at 72°C for 1 minute, and a final step at 72°C for 10 minutes.

The PCR amplifications were performed in an iCycler System (Bio-Rad Laboratories Srl, Segrate, Milan, Italy). Amplicons were detected by electrophoresis of 20 μL of the samples from each PCR tube in a 2% agarose gel in TAE buffer for 2 hours, at 80 V. The amplification products were visualized and photographed under a UV light transilluminator (Gel Doc 2000-Bio-Rad, Milan, Italy) after 30 minutes of ethidium bromide (1 μg/mL) staining. The molecular sizes of the amplicons were determined by comparisons with a commercial DNA molecular weight marker (number VIII; Roche Diagnostics SpA, Milan, Italy). The frequencies of sites positive for each microbiota were recorded.

Statistical analysis

The non-parametric Mann-Whitney U-test was used to evaluate the presence of statistically significant differences among the groups. Results were presented as means ± standard errors (SE), and differences at $P \leq 0.05$ were considered statistically significant.

Results

The clinical parameters for the experimental groups at 20, 30 and 90 days examination are presented in Table 1. The number of sites with presence of bleeding on probing (BOP+) increased significantly after 90 days.

The FMPS and the FMBS remained <20% during the study (data not shown). The number of cultivable oral microflora and members of oral bacteria resident on the healing abutments were examined following either 20, 30, or 90 days of placement in the subject's mouth. Biofilm was detected on all healing abutments after removal from the oral cavity. The results (Fig. 1) indicated a significant increase in the number of cultivable oral flora on the healing screws from 20 days to 30 days ($P = 0.0061$) and to 90 days

Table 1 Number of sites with presence of bleeding on probing (BOP+) in the experimental groups over time.

	20 d	30 d		90 d	
BOP+	0	2	NS	6	S

NS = the difference is not statistically significant; S = difference is statistically significant. (Mann-Whitney U-test test, Wilcoxon paired signed rank test, $P < 0.05$).

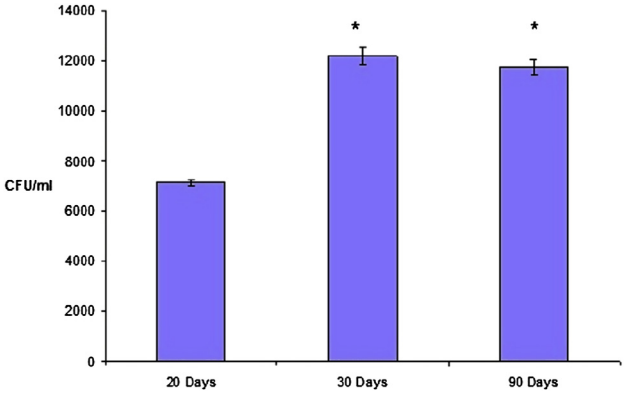

Figure 1 Total bacterial counts (CFU/mL) recovered in the experimental groups (20, 30 and 90 days examinations). Statistically significant differences between the groups (Mann-Whitney Test $P < 0.05$): *$P < 0.05$, statistically significant related to 20 days.

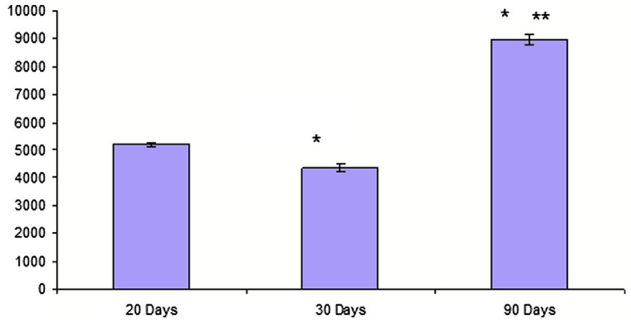

Figure 3 Anaerobic bacteria on the healing abutments over time. Results indicate the mean ± standard error (CFU/mL) of bacteria recovered from 30 healing screws in the clinical trials. Statistically significant differences between the groups (Mann-Whitney Test $P < 0.05$): *$P < 0.05$, statistically significant related to 20 days; **$P < 0.05$, statistically significant related to 30 days.

($P = 0.0061$). No significant differences between the experimental groups at 30 days and 90 days ($P = 0.3429$) were detected and the total bacterial counts remained constant.

After 20 days, the counts of anaerobic oral flora were > aerobic oral flora (Figs. 2 and 3) and at 30 days, the mean counts of anaerobic flora underwent a significant decrease ($P = 0.0061$). After 90 days, the anaerobic counts were significantly greater than 20 and 30 days, ($P = 0.0061$ and $P = 0.0286$, respectively) and constituted the bulk of plaque examined. On the contrary, the aerobic strains after a significant increase at 30 days ($P = 0.0061$), underwent a significant decrease at 90 days ($P = 0.0286$).

The qualitative composition and relative proportions (%) of bacterial species to biofilm grown on healing abutments at 20, 30 and 90 days examinations are summarized in Figs. 4, 5 and 6, respectively. After 20 days, the microflora consisted of mostly Gram-positive bacteria; mainly *Actinomyces* spp. (64.53%), *Streptococcus intermedius* (16.76%) and *Streptococcus* spp. (8.12%) revealed the highest proportions. The microflora analyzed at 30 days, consisted of both Gram-positive and Gram-negative microorganisms and displayed a high variability, as could be seen by the minimal and maximal levels. After 90 days, the presence of both Gram-negative and Gram-positive bacteria remained quite constant with the previous analysis. With regards to the Gram-negative bacteria, *Veillonella* spp. was present with a high percentage level of 19.82%.

No significant differences among the experimental groups were found in the frequencies of detection of monitored bacteria in PCF. At 20 and 30 days of examination, *C. rectus*, *E. corrodens*, *F. nucleatum* and *Porph. gingivalis* were sporadically detected in at each examination. At 90 days, sites with a positive presence for periodontal pathogens increased, but the difference was not significant. *Trep. denticola* was not detected (Table 2) in any examined time interval.

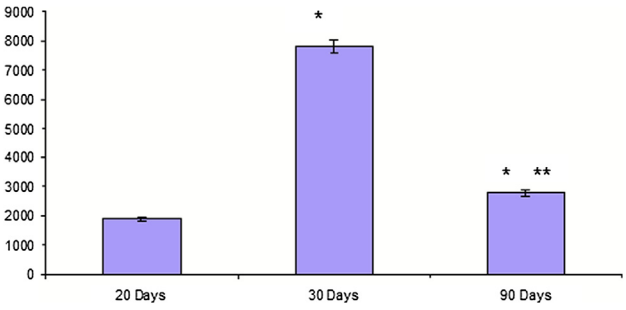

Figure 2 Total aerobic counts of bacteria grown on the healing abutments over time. Results indicate the mean ± standard error (CFU/mL) of bacteria recovered from 30 abutments in the clinical trials. Statistically significant differences between the groups (Mann-Whitney Test $P < 0.05$): *$P < 0.05$, statistically significant related to 20 days; **$P < 0.05$), statistically significant related to 30 days.

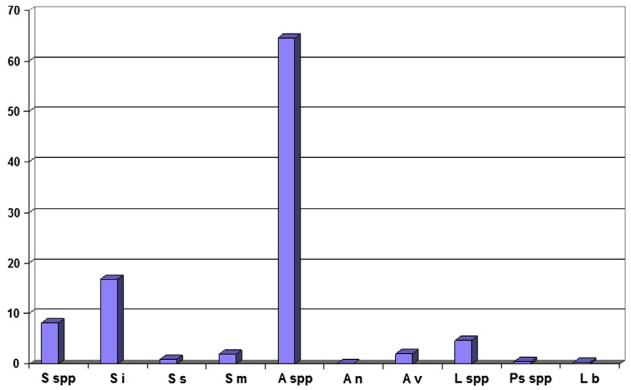

Figure 4 Relative proportions (%) of genera (bacterial species) related to the total bacterial counts grown on healing abutments at 20 days' examination. Sspp = *Streptococcus* spp.; Si = *Streptococcus intermedius;* Ss = *Streptococcus sanguis;* Sm = *Streptococcus mitis;* Aspp = *Actinomyces* spp.; An = *Actinomyces naeslundii;* Av = *Actinomyces viscosus;* Lspp = *Lactobacillus* spp.; Psspp = *Peptostreptococcus* spp.; Lb = *Leptotrichia buccalis*.

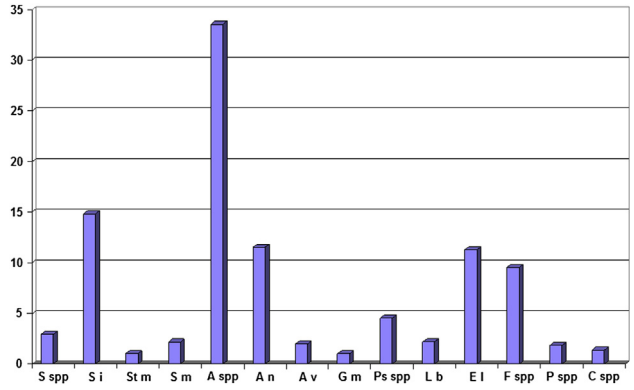

Figure 5 Relative proportions (%) of genera (bacterial species) related to the total bacterial counts grown on healing abutments at 30 days' examination. Sspp = *Streptococcus* spp.; Si = *Streptococcus intermedius;* Stm = *Streptococcus milleri;* Sm = *Streptococcus mitis;* Aspp = *Actinomyces* spp.; An = *Actinomyces naeslundii;* Av = *Actinomyces viscosus;* Gm = *Gemella morbillorum;* Pssp = *Peptostreptococcus* spp.; Lb = *Leptotrichia buccalis;* El = *Eubacterium lentum;* Fspp = *Fusobacterium* spp.; Pspp = *Prevotella* spp.; Cspp = *Capnocytophaga* spp.

Discussion

This study examined the time-dependent microbial colonization of the healing screw *in vivo* and the influence on gingival environments. This anaerobic environment enables formation of the same biofilm as during the development of periodontitis.[11,12]

The periodontal pathogens were detected in none of the analyzed screws. At the same time, it is important to be aware that *Veillonella* spp. are anaerobic Gram-negative cocci and considered to be associated with chronic periodontitis and inflammation of the peri-implant mucosa.[13]

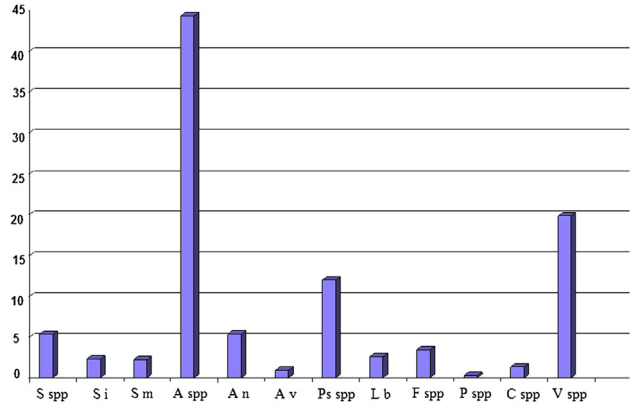

Figure 6 Relative proportions (%) of genera (bacterial species) related to the total bacterial counts grown on healing abutments at 90 days' examination. Sspp = *Streptococcus* spp.; Si = *Streptococcus intermedius;* Sm = *Streptococcus mitis;* Aspp = *Actinomyces* spp.; An = *Actinomyces naeslundii;* Av = *Actinomyces viscosus;* Pssp = Peptostreptococcus spp.; Lb = *Leptotrichia buccalis*; Fspp = *Fusobacterium* spp.; Pspp = *Prevotella* spp.; Cspp = *Capnocytophaga* spp.; Vspp = *Veillonella* spp.

Table 2 Number of sites positive for the presence of each bacterial species, detected by multiplex polymerase chain reaction (PCR), in the experimental groups at 20, 30 and 90 days' examination.

	20	30	90
Aggregatibacter actinomycetemcomitans	ND	ND	1
Campylobacter rectus	1	1	2
Eikenella corrodens	1	2	2
Fusobacterium nucleatum	1	2	4
Porphyromonas gingivalis	ND	1	3
Prevotella intermedia	ND	ND	2
Tannerella forsythia	ND	ND	1
Treponema denticola	ND	ND	ND

ND = no detectable levels. No statistically significant differences were seen. (Fisher's exact test, McNemar's test, $P < 0.05$).

In partially edentulous patients, the composition of the subgingival microbiota is similar to teeth and implants. Transmission of bacteria from residual pockets around neighboring teeth could be possible.[14] In the present study, the subjects maintained good oral hygiene and none of the patients had clinical signs of periodontal disease. We might therefore conclude that the natural teeth cannot influence the surfaces of the healing abutment with the microbiota present on it. Cross infection between healing abutments and natural teeth was unlikely.

Over the time, the screws harbored more complex microbiota, characterized by a lower percentage of coccoid cells and a higher percentage of rod cells. In teeth, these types of microbiota are considered as a more mature plaque.[2]

Qualitative analysis revealed that the periodontal pathogens were present in every sample of PCF. At 90 days, the sites with a positive presence for *A. actinomycetemcomitans*, *C. rectus*, *E. corrodens*, *F. nucleatum*, *Porph. gingivalis*, *Prev. intermedia* and *Tann. forsythia* increased, although the difference was not significant. At the same time, soft issue around dental implants presented clinical signs of inflammation, as demonstrated by the increase of the BOP.

These results reflect the classic features of the peri-implant lesions, in which high levels of periodontal pathogens and superinfecting bacteria were present.[14–17]

One of the causes of peri-implantitis, is the bacterial colonization of the peri-implant pocket. Periodontitis and peri-implantitis are linked to the presence of multiple key pathogens and governed by similar biological parameters. The microorganisms most commonly related to implant failure are: rods and mobile forms of Gram-negative anaerobes (*Prev. intermedia, Porph. gingivalis, A. actinomycetemcomitans, Tann. forsythia, Trep. denticola, Prev. nigrescens, Peptostreptococcus micros, V. parvula* and *F. nucleatum*).[13,18,19]

In this study, the clinical signs of inflammation, in particular BOP+, significantly increased over time. This is an effect of plaque accumulation on healing screws. BOP is a useful clinical parameter for predicting peri-implant and periodontal

''attachment loss''. Luterbacher et al [20] recommended monitoring periodontal and peri-implant tissue status, by the continuous and periodical application of this parameter using a light standardized probing force. The diagnostic power of the BOP was enhanced by the addition of a further microbiological test both on the teeth and on individual implants.[20,21]

The results of this study indicated that maintaining the screws for a period of 90 days caused an important increase in plaque quantity, with a dramatic change in plaque composition. The demonstrated peri-implant inflammation and the presence of periodontal pathogenic bacteria in the peri-implant sulcus, due to marginal plaque accumulation on the healing screws left *in situ* for 90 days, leads to the recommendation that the healing abutments should be removed before this time. Therefore, this should be done during the process of the initial bacterial adhesion, nicknamed a phase of "weak and reversible" binding, and before the establishment of an irreversible attachment. It is therefore very important to develop screw surfaces that reduce the number of initially adhering bacteria, thereby minimizing biofilm formation and subsequent inflammation of the soft tissue.

Otherwise, in cases where the removal of screws is not possible, in light of the possible consequences (peri-implant inflammation), it is crucial to give the patient proper oral hygiene instructions before mounting the healing screws. During the healing period with the screws *in situ*, the patient must be motivated with regular reminders of oral hygiene, and the use of chlorhexidine should be recommended, due to its widely demonstrated bactericidal activity.

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