Canullo Luigi (Orcid ID: 0000-0001-9875-2929)
Pesce Paolo (Orcid ID: 0000-0001-8726-4145)
Caponio Vito (Orcid ID: 0000-0001-5080-5921)
Peñarrocha-Oltra David (Orcid ID: 0000-0001-8419-7645)

Culturomic and qRT-PCR analyses for early contamination of abutments with different surfaces: a randomized clinical trial.

Authors: Luigi Canullo[1], Luca Masucci[2], Gianluca Quaranta [3], Romeo Patini [4] , Vito Carlo Alberto Caponio [5], Paolo Pesce [6] , Andrea Ravidà [7] , David Penarrocha-Oltra [8] , Miguel Penarrocha [9]

1: DDS, PhD, Collaborating Professor of the Master in Oral Surgery and Implantology, University of Valencia. luigicanullo@yahoo.com

2: Department of Microbiology and Virology, Fondazione Policlinico Universitario Agostino Gemelli, Università Cattolica del Sacro Cuore, Largo Agostino Gemelli 1, 00168 Rome (Italy). luca.masucci@unicatt.it

3: Department of Microbiology and Virology, Fondazione Policlinico Universitario Agostino Gemelli, Università Cattolica del Sacro Cuore, Largo Agostino Gemelli 1, 00168 Rome (Italy); gquaranta88@gmail.com

4: DDS, PhD, Institute of Dentistry and Maxillofacial Surgery, Fondazione Policlinico Universitario Agostino Gemelli, Università Cattolica del Sacro Cuore, Largo Agostino Gemelli 1, 00168 Rome (Italy). romeo.patini@unicatt.it

5: DDS, Department of Clinical and Experimental Medicine, University of Foggia, 71122 (Italy). vitocarlo.caponio@unifg.it

6: DDS, PhD, Department of Surgical Sciences (DISC), University of Genoa, Ospedale S. Martino, L. Rosanna Benzi 10, 16132 Genoa Italy. paolo.pesce@unige.it

7: DDS, PhD, Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor, MI, USA ravida@umich.edu

8: DDS, Oral Surgery Unit, Department of Stomatology, Faculty of Medicine and Dentistry, University of Valencia, Valencia (Spain). dpenarrocha@gmail.com

9: DDS, Oral Surgery Unit, Department of Stomatology, Faculty of Medicine and Dentistry, University of Valencia, Valencia (Spain).

L.C. and L.M. contributed equally and share the first authorship.

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Corresponding author:

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Dott. Luigi Canullo
E-mail: luigicanullo@yahoo.com

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Running head: Microbiological analysis of different abutments

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SUMMURY BOX

What is known:

A recent systematic review suggested that a modification of titanium micro-morphology increase connective tissue attachment in the early stage of soft tissue healing. However, an increased surface roughness might be correlated with an increased number of pathogenic bacteria.

What this study adds:

This study suggests that moderately rough surfaces are not associated to a greater microbial adhesion compared to machined ones. Conversely, plasma of argon treated surfaces harbored more bacteria.

Abstract

Background: Rough and/or plasma activated abutments seem to be able to increase soft tissue adhesion and stability, however limited evidence is available about bacterial contamination differences.

Purpose: The aim was to investigate the oral microbiota on four dental abutments with different surfaces by quantitative real-time PCR (qRT-PCR) and culturomic approach.

Methods: Forty patients needing a single implant rehabilitation were involved in the study. 40 healing abutments, especially designed for the study, were divided into four groups according to the surface topography (1. machined (MAC), 2. machined plasma of argon treated (plasmaMAC), 3. Ultrathin Threaded Microsurface (UTM), 4. UTM plasma of argon treated (plasmaUTM). Random assignment was performed according to pre-defined randomization tables. All patients underwent surgical intervention for implant and contextual healing abutment positioning. After 2 months of healing, a sterile cotton swab was used for microbiological sampling for culturomics, while sterile paper points inserted into the sulcus were used for qRT-PCR.

Results: At the end of the study, 36 patients completed all procedures and a total of 36 abutments (9 per group) were analyzed. qRT-PCR retrieved data for 23 bacterial species whereas culturomics revealed the presence of 74 different bacteria, most of them not routinely included into oral cavity microbiological kits of analysis or never found before in the oral microenvironment. No statistically significant differences emerged analyzing the 4 different surfaces (p=0.053). On the contrary higher total and specific bacterial counts were detected in the plasma treated surfaces compared to the untreated ones (p=0,021).

Conclusions: Abutments with different topography and surface treatment resulted contaminated by similar oral bacterial flora. Abutments with moderately rough surface were not associated to a greater bacterial adhesion compared to machined ones. Conversely, more bacteria were found around plasma treated abutments. Furthermore, data reported suggested to include new species not previously sought in the routine analyses of the oral bacterial microflora.

INTRODUCTION

Abutment micro-morphology was demonstrated to dramatically increase connective tissue attachment in the early stage of soft tissue healing[1, 2]. This is because a tridimensional roughened surface seems to prevent epithelial layers from downgrowth, stabilizing the fibrin clot[3]. However, this topographic characterization might increase the risk of contamination[4]. In fact, it was demonstrated that an increased surface roughness might be correlated with an increased number of pathogenic bacteria such as spirochetes[2].

At the same time, different surface treatments have been proposed to reduce pollutants related to manufacturing or abutment customization that could negatively affect the surface energy and the abutment integration[5, 6]. These polluting agents can be found both in customized abutments and in new abutments[7, 8]. Hence, a complete decontamination of the abutment is a prerequisite for its soft tissue integration even if not common among dental community[9]. Different treatments, such as steam vapor or ultrasound baths and UV lights are reported to minimize this contamination[10]. Plasma of argon demonstrated to improve the decontamination effects together with an increase of material surface energy[11]. This led to a decrease of bone resorption/loss[12, 13] , but the increased hydrophilicity, may favor microbiological contamination[14].

Polymerase Chain Reaction (PCR) was discovered by Kary Mullis in 1986 as a consequence of the development of molecular biology in the 1980s. PCR involves the action of an enzyme, polymerase, which is able to synthesize nucleic acids in vitro and replicate a segment of DNA in a semi-conservative way[15]. PCR is currently used in clinical microbiology for the identification of microbial pathogens and its use is normally restricted to the detection of microorganisms with a slow rate of growth or which are almost impossible to cultivate[16]. A variant of PCR, called real-time polymerase chain reaction (real-time PCR) or quantitative polymerase chain reaction (qPCR), consists of a polymerase chain reaction that enables the quantitative determination of the pathogens found[17, 18].

PCR and qPCR have some limitations. One major limitation is that investigators have to know the target sequence because the primers that will allow its selective amplification have to be generated with strict accuracy[19]. Another limitation of PCR analysis is the possible presence of contaminated DNA that, even in the smallest amount, is inevitably amplified, resulting in misleading or ambiguous results[20].

Culturomic analysis, on the other way, is an innovative microbiological technique that consists in the set-up of different culture conditions joined with the identification by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry with the aim of increasing the possibilities of bacterial isolation[21]. Culture conditions are designed with the aim of suppressing the growth of the majority populations, while promoting the selection and growth of microorganisms at lower concentrations. Culturomic was developed with the aim of investigating in a more accurate way the composition of gut microbiota but has recently been proposed also for the investigation of peri-implant microbiome[22]. In this scenario, role of microbiome has gained strong impact in the complex interaction between peri-implant tissue and implant surface, compared to dental surface [23, 24]. Of interest, a systematic review highlighted quantitative characteristics of the peri-implant microbiome may differentially contribute to peri-implantitis, although the existence of still limited evidence on the theme[24, 25]. In this complex scenario, aim of this randomized clinical trial was to investigate if a rough surface abutment and/or a plasma treated surface could harbor more bacteria and/or viruses, by the use of qRT-PCR and culturomic approach. The null hypothesis was that roughened, and plasma activated abutments harbored the same microbial population.

MATERIALS AND METHODS

Study design

The present randomized controlled trial was conducted according to the principles of the Helsinki Declaration at the Oral Surgery Unit of the University of Valencia (Spain). The study protocol was approved by the local Ethical Committee of the University of Valencia (1050031) and patients were required to sign a written consent form after being informed about the purpose of the study. Before the enrollment, a power analysis made prior to the study revealed that 36 abutments would give a power of 90% of detecting a significant difference of 10^3 CFU (colony forming unit) with a standard deviation of 10^2 CFU and an alpha error set to 0.05.

Subject population

In July 2019, periodontally healthy patients[26] previously referred to the department of Oral Surgery and Implantology at the University of Valencia (Valencia, Spain) for implant treatment and waiting for prosthetic rehabilitation, were consecutively screened for study inclusion according to inclusion and exclusion criteria detailed in supplementary table 1. At the start of the study, 40 patients referred to the Department of Oral Surgery and Implantology at the University of Valencia (Valencia, Spain). Of these, 4 were excluded because did not meet the inclusion criteria. At the end of selection process, 36 patients were eligible and enrolled in the study. The total number of patients who observed the follow-up timetable and completed the study resulted to be 36. Periodontal indices (plaque index PI and bleeding on probing BoP) were evaluated for each patient on day of the enrollment.

Surgery

The day of surgery, all patients underwent dental hygiene procedure and chlorhexidine mouthwash 0.20% for 2 minutes. No antibiotic prophylaxis was performed.

Periodontal biotype was evaluated following the definition of Kan et al.: the gingival biotype was categorized as either thin or thick according to the visibility of the underlying periodontal probe through the gingival tissue (visible = thin, not visible = thick) [27].

After local anesthesia a crestal incision was performed and a full thickness flap elevated. Bone level implants with an internal hexagon connection were inserted (Premium ONE, Sweden & Martina, Italy). An especially designed abutment with different microtopography was then screwed with 20N on implants previously inserted and the flaps was repositioned and sutured with a synthetic monofilament to obtain an optimal adaptation of the mucosa around the abutments (Figure 1). No provisional restorations were used. Chlorhexidine 0,12% was prescribe twice a day for the next 6 days. Patients were instructed how to perform oral hygiene, how many times/day they had to brush their teeth, with a regular, commercially available toothpaste and brusher; patients were informed about a healthy diet, poor in sugars, sweets and alcohol.

Randomization

Thirty-six healing abutments, especially designed for the study[4], were used. Eighteen abutments presented a machined surface (MAC), while other 18 presented microgrooves (Ultrathin Threaded Microsurface: UTM) (Figure 2).

All abutments underwent different cleaning processes: sixteen received no treatment (sterile and decontaminated as they come from the industry: MAC and UTM), sixteen were activated by plasma of Argon (Plasma MAC and Plasma UTM).

Implants were randomly connected to one of the four abutments. Random assignment was performed according to pre-defined randomization tables.

A balanced random permuted block approach, ensuring that, at any point in a trial, roughly equal numbers of participants were allocated to all the comparison groups, was used to prepare the randomization tables in order to avoid unequal balance among the four treatment groups. Allocation concealment was maintained by means of opaque sealed envelopes, opened at the time of reopening containing sterile envelops with the tested abutments.

Activation process

Abutments belonging to Plasma MAC or Plasma UTM groups underwent Argon plasma treatment in a plasma reactor (Diener Electronic GmbH, Jettingen, Germany). The treatment conditions were 75

W of power and -10MPa of pressure for 12 minutes. Cleaning processes were performed immediately before abutment connection.

Samples Collection

Two months after surgery, patients were recalled for oral microbiome sampling.

During this period, patients were asked to not use chlorhexidine or any antibiotics and to regularly perform home dental hygiene brushing teeth three times a day and performing interdental hygiene.

Patients were asked to brush their teeth early in the morning of their appointment and not to eat, drink or perform any oral hygiene procedure for at least two hours before sample collection.

Sampling was performed through a sterile eSwab (COPAN, Murrieta, USA), put in contact circumferentially in the peri-implant sulcus for the collection of plaque for culturomic analysis.

At the same time, 5 sterile paper points[i] were gently inserted in the peri-implant sulcus for qRT-PCR analysis for 20 seconds, according to Canullo et al[28].

Bleeding on probing (BoP) and plaque index (PI) were assessed following the definition of Ainamo and Bay and Silness and Loe, respectively at the bacterial sample collection visit (4 points each implant) [29, 30].

qRT-PCR analysis

Methodology regarding microbiological analyses followed a previously published procedure[28]. In addition to the 21 species previously studies, the following new bacteria have been investigated in this article since qRT-PCR was also carried out: *Parvimonas micra* (Pm), *Prevotella nigrescens* (Pn), *Campylobacter gracilis* (Cg), *Capnocytophaga ochracea* (Co), *Capnocytophaga sputigena* (Cs), *Campylobacter concisus* (Cc), *Streptococcus mitis* (Smitis), *Streptococcus spp.*(Sspp), *Actinomyces odontolyticus* (Ao), *Actinomyces viscosus* (Av), *Veillonella parvula* (Vp), *Enterocccus spp.* (Espp) and

Enterococcus faecalis (Ef). List of investigated bacterial species is reported in supplementary table 2.

Total bacterial DNA extraction and quantitative real-time PCR assays

Total DNA was isolated by Institut Clinident SAS (Aix en Provence, France) using the QIAcube® HT Plasticware and QIAamp® 96 DNA QIAcube® HT Kit (Qiagen, Hilden, Germany) ‡ according to manufacturer's guidelines. The elution volume used in this study was 150 µl.
qRT-PCR analysis for *Entamoeba histolytica*, *Epstein Barr* virus, *Cytomegalovirus* and *Herpes* virus type 1 and 2 was carried out by Espace Lab, Geneva, Swizerland.

Culturomics protocol and bacterial identification

Each sample was enriched with 5 ml of Brucella Broth ‖ and then centrifuged at 3500 g for 15 minutes. Supernatant was discarded and the pellet was resuspended in 5 mL of Brucella Broth. Suspension was divided in two aliquots and inoculated in two bottles of pediatric blood cultures §. At this point, one bottle was incubated with shaking for 10 days at 37˚C whereas the second one for 10 days at 42˚C. After ten days of incubation, 100 µl of suspension, from both bottles, were plated on the following agar media: Trypticase Soy Agar II (BECTON-DICKINSON, Heidelberg, Germany), Columbia CNA Agar with 5% Sheep Blood for aerobes detection §; Chocolate agar PolyViteX for fastidous bacteria (BIOMÉRIEUX, Marcy-l'Étoile, France); Schaedler Agar with Vitamin K1 and 5% Sheep Blood and CNA agar for anaerobes. Microae[ii]rophilic and anaerobic conditions were guaranteed by a pouch system CampyGenTM and Concept anaerobic workstation ** respectively. Media and culture conditions are summarized in supplementary table 3. Subsequently, each colony was sub-cultivated in order to obtain a pure culture for bacterial identification.
Colonies identification was performed by mass spectrometry (MALDI-TOF), which allows a rapid and effective identification. The MALDI-TOF principle corresponds to a soft ionization mechanism, which is obtained by using a matrix added to the bacterial colonies on metal plates. For plate extraction, 1 to 4 colonies from subcultures were spotted on the target plate. Each spot was overlaid with 1 µl of alpha-cyano-4-hydroxycinnamic acid (α-CHCA). Spectra were acquired from 1000 laser shots at a minimum intensity threshold of 30 arbitrary units (A.U.). Ionization was achieved by using a UV laser beam. The measurement of the time of flight into the tube to reach a detector generated spectra. Spectral comparison with data from the defined database available was automatically performed and permitted identification.

Statistical analysis

qRT-PCR analysis results for microbial expression were obtained as continuous data, while culturomic results as dichotomous. For qRT-PCR, differences among the 4 groups were investigated with the Kruskal-Wallis test and Bonferroni post-hoc analysis. In addition, a comparative analysis of plasma treated (Plasma UTM + Plasma MAC) vs machined (MAC and UTM) surfaces was performed by means of the Mann-Whitney test. While for culturomics, differences among groups were performed with the Fisher's exact test. All the analyses were performed with the software Stata 16.0 (StataCorp, Texas 77845 USA).

Differences among demographic characteristics of patients and type of abutment were investigated through Chi-square test or Mann-Whitney. Moreover, we performed a two-way ANOVA to deepen the link among the type of abutment, plaque index and total count of bacteria or red bacterial complex of Socransky.

In order to investigate any possible relation between Plaque Index and the total bacterial count sampled, we performed Spearman rank correlation test.

RESULTS

At the end of the study, 36 patients requiring single implant rehabilitation completed the trial. Indeed, 4 patients were excluded due to antibiotic intake during the healing phase.

Clinical characteristics of patients included in this study are reported in Table 1. There was no difference in mean age of patients within all four kinds of abutment and when comparing plasma vs non-plasma treated abutments (Kruskal-Wallis, p-value=0.162; Mann-Whitney, p-value=0.788). Chi-Square test failed to show any statistically significant difference between sex of patients and type of abutment (p-value=0.098) and for plasma or non-plasma treated classification (p-value=0.502). Difference in both PI and BoP were investigated among patients with all four kind of abutment and also comparing plasma vs non-plasma treatment groups (Kruskal-Wallis test, PI – all 4 kind of abutments p-value=0.252; PI – plasma vs non-plasma abutment Mann-Whitney test, p-value=0.059; Kruskal-Wallis test, BoP – all 4 kind of abutments p-value=0.351 and BoP – plasma vs non-plasma abutment Mann-Whitney test, p-value=0.719). At last, Chi-square test did not show any difference among abutment group and biotype (p-value=0.801 for all four kind of abutments; p-value=0.480 for plasma or non-plasma treated abutments).

Totally, 74 bacterial species were isolated from culturomic analysis (Supplementary Table 4). Some species never detected before in the oral cavity were identified: *Acidaminococcus fermentans, Acidaminococcus intestinii, Peptoniphilus harei* and *Peptoniphilus lacrimalis*. Mean quantification of the isolated species through qRT-PCR for the four different abutments is reported in table 2. Figure 3 shows the difference of relative frequencies of Socransky bacterial complexes among the different kind of abutments (Figure 3a) and comparing plasma- vs plasma treated abutments (Figure 3b). qRT-PCR analysis detected the presence of Epstein Barr Virus (EBV) in 2 patients (35 copies/µl and 0.3 copies/µl) and of Herpes Simplex Virus (HSV1-2) in one patient (1.5 copies/µl).

Results from Kruskal-Wallis analysis and Bonferroni post-hoc comparison did not show any difference among bacterial species quantified through qRT-PCR and type of abutment (Results not shown). In order to assess the role of plasma treatment, qRT-PCR data were furtherly analyzed by looking at mean difference between the group of plasma-treated abutments vs non-plasma ones. Mean quantification of each bacterial species composition in those groups is collected in table 3. Bacterial total count resulted next to significant level showing a higher number in plasma treated abutments (Mann-Whitney test, p-value=0.059 – Plasma=$1.0*10^9$ vs non plasma=$2.4*10^8$). Similar results were also obtained when looking at single bacterial species. Specifically, Fusobacterium

Nucleatum (FN) and Eikenella Corrodens (EC) were higher in patients with plasma treatment abutment, next to significant level difference (Table 3). Of interest, Streptococcus Mitis (SM) resulted statistically significant higher in the plasma-group (Mann-Whitney test, p-value=0.044 – Plasma=$1.2*10^5$ vs non plasma=$1.4*10^4$), while Lactobacillus species (Lacto spp.) prevailed in non-plasma group (Mann-Whitney test, p-value=0.016 – Plasma=$1.0*10^5$ vs non plasma=$2.5*10^6$).

Furtherly, a two-way ANOVA was conducted that examined the effect of PI (ordinal variable) and typology of abutment on total count of bacteria and, specifically, on red-complex of Socransky. There was a statistically significant interaction between the effects of abutment type and PI on total bacterial count (Two-way ANOVA interaction p-value=0.034). Simple main effects analysis showed that both, types of abutment and PI contributed to the bacterial total count (respectively p-value=0.008; p-value<0.001). Same results were obtained when comparing plasma vs non-plasma treated implants (Two-way ANOVA interaction model p-value=0.044; PI p-value=0.002; plasma vs no-plasma treated abutment p-value=0.019).

Although, this difference seems to be influenced by the Plaque Index, Spearman rank test did not show any significant correlation between the total amount of bacteria and PI (p-value=0.435; ρ=0.134).

Because red bacterial complex of Socransky collects most of periodontal pathogenic bacteria, the same statistical tests to investigate any possible link to both PI and typology of abutment was applied. Two-way ANOVA analysis failed to show any statistically significant result for all variables, such as the model interaction (p-value=0.182), PI (p-value=0.199) and typology of abutment (p-value=0.319). Same result was obtained when plasma vs non-plasma treatments were compared (Two-way ANOVA interaction model p-value=0.621; PI p-value=0.234; plasma vs no-plasma treated abutment p-value=0.565).

Of interest, a higher number of different microbial species was found on patients with thin periodontal biotype, such total microbial count, *Capnocytophaga sputigena* (CS), *Campylobacter Rectus* (CR), *Eikenella Corrodens* (EC), *Veillonella Parvula* (VP) and Enterococcus spp.

DISCUSSION

The aim of this RCT was to evaluate differences in microbial composition of oral microflora colonizing the soft tissue/abutment interface using different surface abutments, subjected or not to a further activation treatment.

The null hypothesis was that roughened and plasma activated abutments harbored the same microbial population. To validate this hypothesis, two microbiological analyses were carried out: qRT-PCR and culturomic analysis. Data reported in the present study rejected the null hypothesis. When comparison was run not considering different surfaces but only the bio-activation, slightly more (total count) bacteria were found around plasma activated surfaces. This treatment might result in an improved abutment surface energy and wettability. As a consequence, an amplified microbial contamination occurred. This is in agreement with a previously published study, which showed how exposed bio-activated surfaces could allow an increased marginal microbiological contamination compared to non-treated surfaces[14].

However, the clinical relevance of this peripheral contamination could be questioned. Indeed, in standard clinical conditions, prosthesis crown covers the marginal portion of the abutment, so that the microbial growth is allowed only along the peri-implant sulcus, where the biofilm is counteracted by the connective tissue directly in contact with the abutment surface[31]. Although additional studies might be requested to clarify the competition between the microbiological and the connective components, clinical results of recently published studies may suggest the evolution of this competition[32]. However, improved outcomes, in terms of marginal bone level changes even after 5-year follow-up, were observed in favor of plasma activated abutments by argon in both healthy and patients with history of periodontitis, despite the risk of a higher microbial contamination on activated abutment due to the increased surface energy[33, 34].

Additionally, it must be noted that all cautions to prevent abutment contamination preoperatively and during the abutment connection phase were used: abutment decontamination and sterilization[8] and preoperative mouth disinfection procedures[35].

At the same time, adoption of a prosthetic workflow preventing abutment from disconnections (using a torque value of 20N), allowed an undisturbed soft tissue healing, preventing further bacteria contamination of the metallic surfaces.

qRT-PCR provided data regarding 23 bacterial species and the statistical analysis carried out showed that patients with a thin periodontal biotype were colonized by a total count of bacteria higher than patients with a thick biotype.

Abutments in this study were divided into groups: smooth and with moderate surface roughness (0.6 micron)[1]; each group was further divided according to the surface addictive treatment or not using Argon-based plasma; this treatment has been shown to enhance peri-implant soft tissue healing in early stage, with an in vitro microbial inactivation/sterilization potential associated with the preservation of the marginal bone level[36].

However, the molecular analysis performed by qRT-PCR only detected slight statistically significant difference among the 4 abutments, limited to few species, such as FN and EC, which were higher in patients with plasma treatment abutment (Table 3). Of interest, Streptococcus Mitis (SM) resulted statistically significant higher in the plasma-group, while Lactobacillus species (Lacto spp.) prevailed in non-plasma group.

Culturomic analysis revealed variable prevalence of 74 different species. This led to the identification of new species which may colonize the oral cavity, confirming the presence of *Olsenella uli, Slackia exigua, Solobacterium moorei* and bacteria belonging to the *Selenomonas* genus that have recently been identified as new pathogens belonging to the dento-periodontal microbiota[36, 37]. Another interesting finding is the presence of bacteria belonging to the *Atopobium* genus whose clinical significance has not been clearly defined to date. This could be due to the fact that these bacteria are not yet included in the commercially available bacterial systems and, therefore, are likely to be mistakenly identified as *Lactobacillus* or *Streptococcus* spp. based on the morphological characteristics of their colonies[38]. Culturomic analysis also highlighted some species never detected before in the oral cavity: *Acidaminococcus fermentans, Acidaminococcus intestinii, Peptoniphilus harei* and *Peptoniphilus lacrimalis*. Such bacteria are difficult to recover with usual clinical cultures. Indeed, they have been reported only with the most widespread use of 16S genome sequencing analysis and MALDI-TOF for identification[39]. Although culturomic analysis did not allow intrinsically a quantification, these results address future clinical research towards the quantification of species that are not traditionally associated with the oral cavity or that have only been recently detected. This is the case of the pulmonary pathogen *Pseudomonas aeruginosa* whose presence in the oral cavity has already been demonstrated previously. In fact, Rivas Caldas et al. showed that the populations that compose the pulmonary microbiota can easily move through the trachea, colonize the oral cavity and there be hosted forming a bacterial reservoir[40].

Results of viral component of the oral microbiota showed that only a small percentage of patients was positive for the presence of EBV (2 patients) and HSV1-2 (31 patient). Information regarding EBV colonization in periodontal health and disease are sometimes contradictory since EBV has been

detected only in a minor part of patients in periodontal health status[41] whereas other studies revealed no presence[42].

Limitations of the present preliminary study are strictly related to its observational design. In fact, it would be interesting analyzing the interaction between abutment integration (ratio between epithelium and connective tissue components around the abutment) and the presence of specific bacteria. Additionally, short follow-up does not allow drawing any relevant longitudinal observation on the microbiological contamination of the abutment. For this purpose, a supplementary study is strongly suggested. A further limitation has been the impossibility of recording changes in the microbial flora of patients over time even though they observed a rigorous follow-up protocol. In fact, further microbiological analyses would have excessively increased research costs. An important limitation of culturomics, however, must be highlighted, since it is based on the induction of the growth of the greatest number of bacterial species existing in the sample, therefore its analytical result can only be qualitative. Such limitation, anyhow, can be avoided by combining culturomics with the qRT-PCR even if it causes an increase in costs.

At the same time, preoperative and longitudinal comparisons of the microbiological data might give a clear overview of how different surfaces (activated vs not, smooth vs rough) behave longitudinally. Although more bacteria, intended as total count, were found around plasma treated abutments, a study from Garcia et al.[31] showed that these kinds of abutments are characterized by an improved soft tissue-abutment integration. Whereas how these factors may differentially contribute to the therapy success, has to be addressed in future studies. While microbiological analysis, showing different amounts of bacteria, could not be clinically relevant, histological based study, integrating information from this study, are able to evaluate the tissue-abutment health status, with clinical consequences on type/treatment of abutment to choose.

CONCLUSIONS

This study showed that abutments with different topography and different surface energy treatment might be contaminated by similar oral microbial flora when a correct surgical-prosthetic protocol is adopted. Abutments with moderately roughed surface were not associated to a greater microbial adhesion compared to machined ones. Conversely, when abutment types were aggregated according to the treatment, slightly more bacteria were found around plasma treated abutments.

However, the clinical effect of this amplified microbial contamination should be tested by histological analysis. Furthermore, culturomic analysis showed that the oral cavity is colonized by bacterial species not detected so far, some coming from other anatomical districts. This finding might suggest the inclusion of new species not previously sought in the routine analyses of the oral microbiota.

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All authors report no conflicts of interest related to this study.

FIGURE LEGENDS

Figure 1. Different phases of the surgical intervention (a: preoperative, b: post-surgery when dental implant was installed, and dental abutment was positioned in the same surgical phase, c: healing phase 2 months after surgery).

Figure 2. Microphotograph (30x and 250x) of the abutments used in the study with detail of the different surfaces. A: machined surface (MAC). B: Ultrathin Threaded Microsurface (UTM).

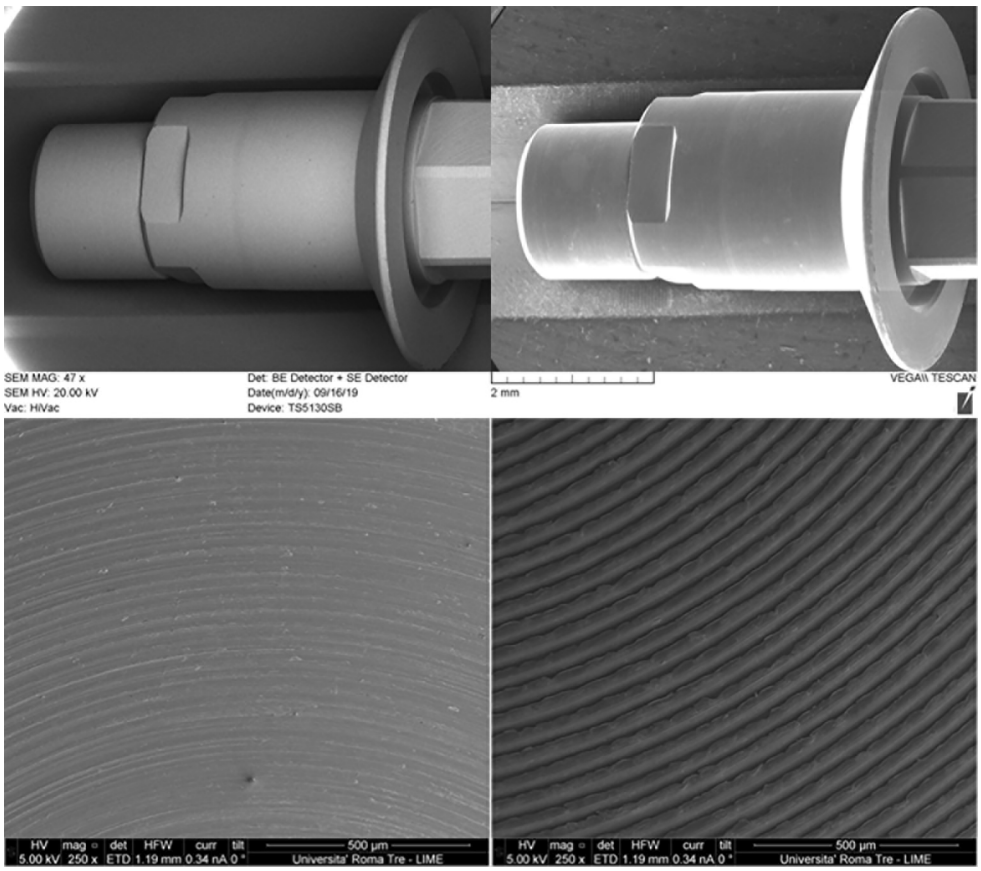
Figure 3. Histograms showing differential percentages of Socransky complex among different kind of material/treatment abutments. a) Showing differences among MAC – Plasma Mac – UTM – Plasma UTM abutments; b) Showing differences of non-plasma vs plasma treated abutments.

REFERENCES

1. Pesce P, Menini M, Tommasato G, Patini R, Canullo L. Influence of modified titanium abutment surface on peri-implant soft tissue behaviour: A systematic review of histological findings. *Int J Oral Implantol (Berl)* 2019; **12**: 419-429.
2. Canullo L, Menini M, Santori G, Rakic M, Sculean A, Pesce P. Titanium abutment surface modifications and peri-implant tissue behavior: a systematic review and meta-analysis. *Clin Oral Investig* 2020; **24**: 1113-1124.
3. Piattelli A, Pontes AE, Degidi M, Iezzi G. Histologic studies on osseointegration: soft tissues response to implant surfaces and components. A review. *Dent Mater* 2011; **27**: 53-60.
4. Canullo L, Genova T, Gross Trujillo E, Pradies G, Petrillo S, Muzzi M, Carossa S, Mussano F. Fibroblast Interaction with Different Abutment Surfaces: In Vitro Study. *Int J Mol Sci* 2020; **21**.
5. Neoh KG, Hu X, Zheng D, Kang ET. Balancing osteoblast functions and bacterial adhesion on functionalized titanium surfaces. *Biomaterials* 2012; **33**: 2813-2822.
6. Att W, Hori N, Takeuchi M, Ouyang J, Yang Y, Anpo M, Ogawa T. Time-dependent degradation of titanium osteoconductivity: an implication of biological aging of implant materials. *Biomaterials* 2009; **30**: 5352-5363.
7. Sawase T, Wennerberg A, Hallgren C, Albrektsson T, Baba K. Chemical and topographical surface analysis of five different implant abutments. *Clin Oral Implants Res* 2000; **11**: 44-50.
8. Canullo L, Micarelli C, Iannello G. Microscopical and chemical surface characterization of the gingival portion and connection of an internal hexagon abutment before and after different technical stages of preparation. *Clin Oral Implants Res* 2013; **24**: 606-611.
9. Canullo L, Tallarico M, Chu S, Penarrocha D, Ozcan M, Pesce P. Cleaning, Disinfection, and Sterilization Protocols Employed for Customized Implant Abutments: An International Survey of 100 Universities Worldwide. *Int J Oral Maxillofac Implants* 2017; **32**: 774-778.
10. Canullo L, Micarelli C, Lembo-Fazio L, Iannello G, Clementini M. Microscopical and microbiologic characterization of customized titanium abutments after different cleaning procedures. *Clin Oral Implants Res* 2014; **25**: 328-336.
11. Noopan S, Unchui P, Techotinnakorn S, Ampornaramveth RS. Plasma Sterilization Effectively Reduces Bacterial Contamination in Dental Unit Waterlines. *Int J Dent* 2019; **2019**: 5720204.
12. Canullo L, Penarrocha D, Micarelli C, Massidda O, Bazzoli M. Hard tissue response to argon plasma cleaning/sterilisation of customised titanium abutments versus 5-second steam cleaning: results of a 2-year post-loading follow-up from an explanatory randomised controlled trial in periodontally healthy patients. *Eur J Oral Implantol* 2013; **6**: 251-260.
13. Canullo L, Penarrocha D, Clementini M, Iannello G, Micarelli C. Impact of plasma of argon cleaning treatment on implant abutments in patients with a history of periodontal disease and thin biotype: radiographic results at 24-month follow-up of a RCT. *Clin Oral Implants Res* 2015; **26**: 8-14.
14. Canullo L, Penarrocha-Oltra D, Marchionni S, Bagan L, Penarrocha-Diago MA, Micarelli C. Soft tissue cell adhesion to titanium abutments after different cleaning procedures: preliminary results of a randomized clinical trial. *Med Oral Patol Oral Cir Bucal* 2014; **19**: e177-183.
15. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. 1986. *Biotechnology* 1992; **24**: 17-27.
16. Wolk D, Mitchell S, Patel R. Principles of molecular microbiology testing methods. *Infect Dis Clin North Am* 2001; **15**: 1157-1204.

17. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009; **55**: 611-622.
18. Logan JM, Edwards KJ, Saunders NA, Stanley J. Rapid identification of Campylobacter spp. by melting peak analysis of biprobes in real-time PCR. *J Clin Microbiol* 2001; **39**: 2227-2232.
19. Garibyan L, Avashia N. Polymerase chain reaction. *J Invest Dermatol* 2013; **133**: 1-4.
20. Schochetman G, Ou CY, Jones WK. Polymerase chain reaction. *J Infect Dis* 1988; **158**: 1154-1157.
21. Chen K, Pachter L. Bioinformatics for whole-genome shotgun sequencing of microbial communities. *PLoS Comput Biol* 2005; **1**: 106-112.
22. Martellacci L, Quaranta G, Patini R, Isola G, Gallenzi P, Masucci L. A Literature Review of Metagenomics and Culturomics of the Peri-implant Microbiome: Current Evidence and Future Perspectives. *Materials (Basel)* 2019; **12**.
23. Shahabouee M, Rismanchian M, Yaghini J, Babashahi A, Badrian H, Goroohi H. Microflora around teeth and dental implants. *Dent Res J (Isfahan)* 2012; **9**: 215-220.
24. Pokrowiecki R, Mielczarek A, Zareba T, Tyski S. Oral microbiome and peri-implant diseases: where are we now? *Ther Clin Risk Manag* 2017; **13**: 1529-1542.
25. Rakic M, Grusovin MG, Canullo L. The Microbiologic Profile Associated with Peri-Implantitis in Humans: A Systematic Review. *Int J Oral Maxillofac Implants* 2016; **31**: 359-368.
26. Caton JG, Armitage G, Berglundh T, Chapple ILC, Jepsen S, Kornman KS, Mealey BL, Papapanou PN, Sanz M, Tonetti MS. A new classification scheme for periodontal and peri-implant diseases and conditions - Introduction and key changes from the 1999 classification. *J Clin Periodontol* 2018; **45 Suppl 20**: S1-S8.
27. Kan JY, Morimoto T, Rungcharassaeng K, Roe P, Smith DH. Gingival biotype assessment in the esthetic zone: visual versus direct measurement. *Int J Periodontics Restorative Dent* 2010; **30**: 237-243.
28. Canullo L, Penarrocha-Oltra D, Soldini C, Mazzocco F, Penarrocha M, Covani U. Microbiological assessment of the implant-abutment interface in different connections: cross-sectional study after 5 years of functional loading. *Clin Oral Implants Res* 2015; **26**: 426-434.
29. Ainamo J, Bay I. Problems and proposals for recording gingivitis and plaque. *Int Dent J* 1975; **25**: 229-235.
30. Silness J, Loe H. Periodontal Disease in Pregnancy. Ii. Correlation between Oral Hygiene and Periodontal Condtion. *Acta Odontol Scand* 1964; **22**: 121-135.
31. Irshad M, Scheres N, Crielaard W, Loos BG, Wismeijer D, Laine ML. Influence of titanium on in vitro fibroblast-Porphyromonas gingivalis interaction in peri-implantitis. *J Clin Periodontol.* 2013; **40**:841-9.
32. Garcia B, Camacho F, Tallarico M, Perez S, Penarrocha-Oltra D, Canullo L. Soft tissue cell adhesion to titanium abutments after different cleaning procedures: results of a randomized clinical trial. *Clin Oral Implants Res* 2017;**28**:1269-1277.
33. Canullo L, Tallarico M, Penarrocha-Oltra D, Monje A, Wang HL, Penarrocha-Diago M. Implant Abutment Cleaning by Plasma of Argon: 5-Year Follow-Up of a Randomized Controlled Trial. *J Periodontol* 2016; **87**: 434-442.
34. Canullo L, Tallarico M, Penarrocha M, Corrente G, Fiorellini J, Penarrocha D. Plasma of Argon Cleaning Treatment on Implant Abutments in Periodontally Healthy Patients: Six Years Postloading Results of a Randomized Controlled Trial. *Int J Periodontics Restorative Dent* 2017; **37**: 683-690.
35. Quirynen M, Mongardini C, de Soete M, Pauwels M, Coucke W, van Eldere J, van Steenberghe D. The role of chlorhexidine in the one-stage full-mouth disinfection treatment of

patients with advanced adult periodontitis. Long-term clinical and microbiological observations. *J Clin Periodontol* 2000; **27**: 578-589.
36. Mombelli A. Microbial colonization of the periodontal pocket and its significance for periodontal therapy. *Periodontol 2000* 2018; **76**: 85-96.
37. Hiranmayi KV, Sirisha K, Ramoji Rao MV, Sudhakar P. Novel Pathogens in Periodontal Microbiology. *J Pharm Bioallied Sci* 2017; **9**: 155-163.
38. Brown K, Church D, Lynch T, Gregson D. Bloodstream infections due to Peptoniphilus spp.: report of 15 cases. *Clin Microbiol Infect* 2014; **20**: O857-860.
39. D'Auria G, Galan JC, Rodriguez-Alcayna M, Moya A, Baquero F, Latorre A. Complete genome sequence of Acidaminococcus intestini RYC-MR95, a Gram-negative bacterium from the phylum Firmicutes. *J Bacteriol* 2011; **193**: 7008-7009.
40. Rivas Caldas R, Le Gall F, Revert K, Rault G, Virmaux M, Gouriou S, Hery-Arnaud G, Barbier G, Boisrame S. Pseudomonas aeruginosa and Periodontal Pathogens in the Oral Cavity and Lungs of Cystic Fibrosis Patients: a Case-Control Study. *J Clin Microbiol* 2015; **53**: 1898-1907.
41. Canullo L, Pesce P, Botticelli D, Covani U, Jankovic S, Jovanovic T, Rakic M. What is the Impact of Epstein-Barr Virus in Peri-implant Infection? *Int J Oral Maxillofac Implants* 2018; **33**: 58-63.
2. Kazi M, Bharadwaj R. Role of herpesviruses in chronic periodontitis and their association with clinical parameters and in increasing severity of the disease. *Eur J Dent* 2017; **11**: 299-304.



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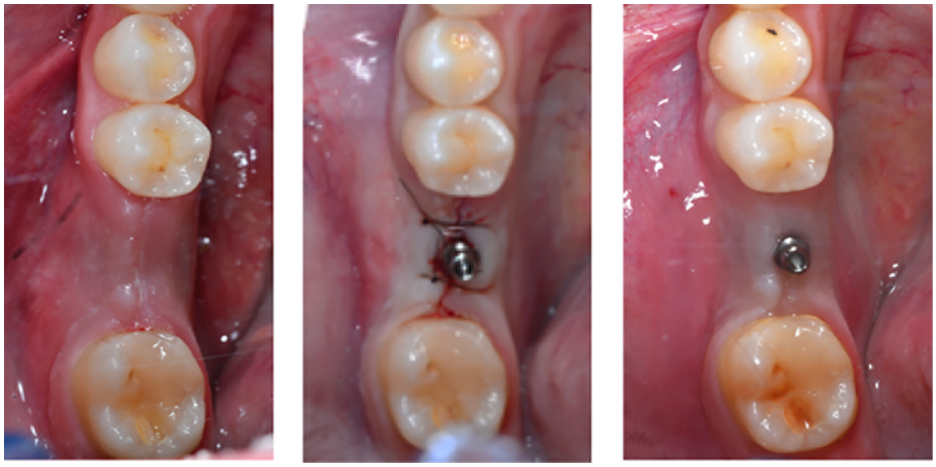

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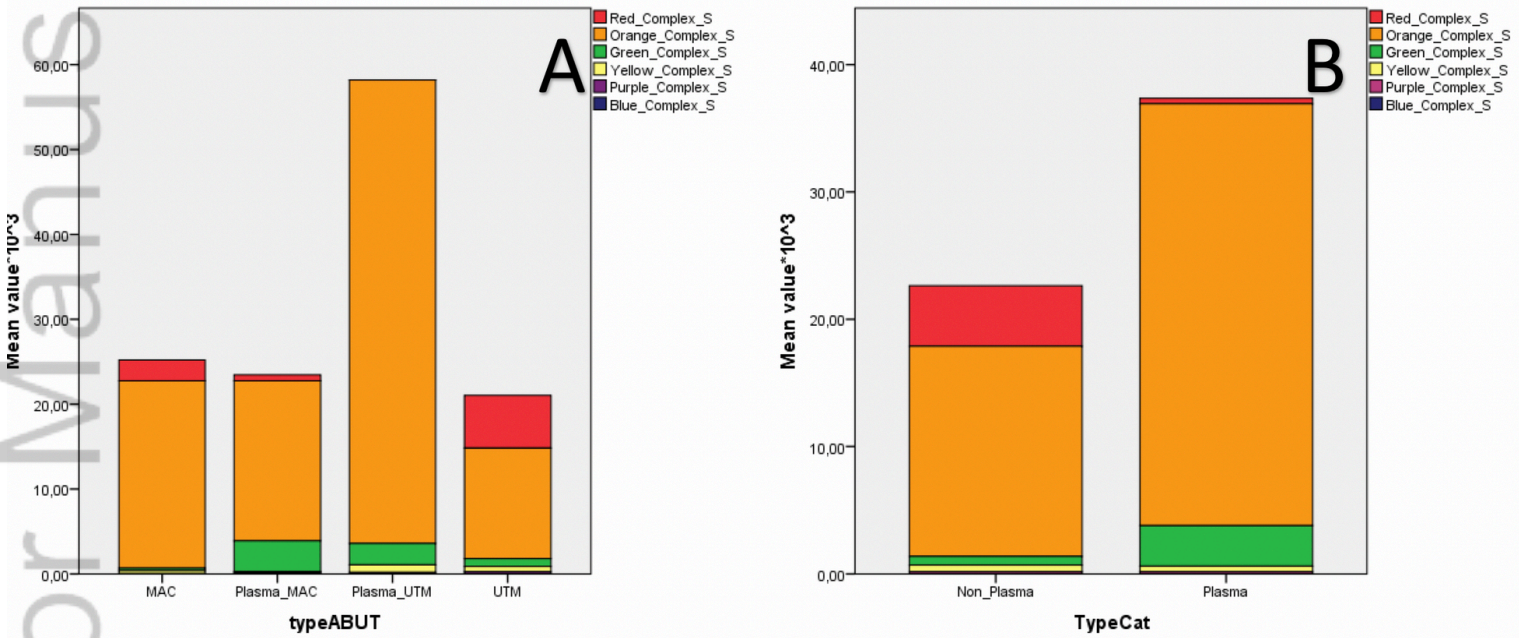

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Table 1. Demographic and clinical characteristics of the enrolled patients.

	Abutments (n = 36)				*p*-value
	PLASMA MAC (n = 9)	MAC (n = 9)	UTM (n = 9)	PLASMA UTM (n = 9)	
Sex (n)					
Male	8 (88.9%)	5 (55.6%)	4 (44.4%)	3 (33.3%)	
Female	1 (11.1%)	4 (44.4%)	5 (55.6%)	6 (66.7%)	.098 - NS
Age (y)	53 ± 14 (26-79)	62 ± 11 (46-74)	48 ± 5 (44-56)	55 ± 17 (29-81)	.162 - NS
Periodontal and peri-implant parameters					
Healthy	8/9 (88.9%)	9/9 (100%)	9/9 (100%)	8/9 (88.9%)	
History of periodontitis	1/9 (11.1%)	0	0	1/9 (11.1%)	.537 - NS
Biotype					
Thin	4/9 (44.4%)	3/9 (33.3%)	2/9 (22.2%)	3/9 (33.3%)	
Thick	5/9 (55.6%)	6/9 (66.7%)	7/9 (77.8%)	6/9 (66.7%)	.801 - NS
BoP*	1 ± 1.22	0.78 ± 1.39	1.11 ± 1.36	0.33 ± 0.70	.351 - NS
PI†	1.22 ± 1.39	2.11 ± 1.83	2 ± 1.22	1.22 ± 1.64	.252 - NS

Data are given as number of abutments or patients and percentage (in parenthesis) or as mean values and standard deviations.
* = Bleeding on probing; † = Plaque Index; NS = not significant.

Table 2. Mean values of total count and different bacterial species from qRT-PCR analysis, comparing among all four kind of abutments (MAC; PLASMA-MAC; UTM and PLASMA-UTM).

Group	Total Count	Aa	Pg	Tf	Td	Pi	Pm	Fn	Cr	Ec
MAC	$2.07*10^8$	////	$2.4*10^5$	$7.9*10^3$	$8.9*10^4$	$2.2*10^6$	$3.0*10^6$	$1.5*10^6$	$1.7*10^5$	$3.7*10^5$
PLASMA MAC	$8.10*10^8$	$2.6*10^3$	$3.2*10^4$	$1.8*10^4$	$3.3*10^5$	$1.1*10^6$	$9.8*10^5$	$9.8*10^6$	$1.5*10^6$	$1.0*10^7$
UTM	$2.78*10^8$	/////	$1.6*10^6$	$1.1*10^5$	$2.5*10^5$	$1.5*10^6$	$1.3*10^6$	$2.5*10^6$	$8.4*10^5$	$4.6*10^5$
PLASMA UTM	$1.36*10^9$	/////	$8.5*10^6$	$3.2*10^5$	$2.1*10^5$	$2.3*10^7$	$1.0*10^7$	$8.0*10^6$	$1.2*10^6$	$3.4*10^6$
p-value	0.146	0.392	0.750	0.166	0.632	0.158	0.225	0.268	0.327	0.266

Pn	Cg	Co	Cs	Cc	Sm	Strepto spp	Av	Ao	Vp	Entero spp	Ef	Lacto spp.	S. mutans
/////	/////	$2*10^3$	$2.8*10^3$	$1.9*10^2$	$2*10^4$	$1.7*10^5$	/////	$4.1*10^3$	$3.4*10^3$	$1.7*10^3$	/////	$2*10^5$	/////
2.4**$*10^2$**	/////	$1.5*10^3$	$5.6*10^4$	$2.9*10^2$	$6.9*10^4$	$2.1*10^5$	$3.5*10^3$	$1.3*10^4$	$1.0*10^5$	$1.9*10^3$	/////	$3.9*10^5$	/////
$5.3*10^2$	$2.2*10^3$	$3.2*10^4$	$4.2*10^3$	$1.5*10^2$	$8.6*10^3$	$4.4*10^4$	$2.6*10^3$	$9.2*10^3$	$1.6*10^5$	$1.9*10^2$	/////	$2.3*10^4$	$8.1*10^3$
/////	/////	$3.5*10^6$	$4.7*10^5$	$3.4*10^2$	$1.8*10^5$	$1.5*10^6$	$2.4*10^3$	$5.9*10^4$	$2.8*10^5$	$4.2*10^4$	/////	$4.2*10^6$	/////
0.584	0.465	0.887	0.267	0.996	0.133	0.258	0.581	0.789	0.314	0.276	1	0.111	0.392

Table 3. Mean values of total count and different bacterial species from qRT-PCR analysis, comparing plasma vs non-plasma abutment groups (PLASMA-UTM AND PLASMA MAC vs UTM and MAC).

Group	**Total Count**	**Aa**	**Pg**	**Tf**	**Td**	**Pi**	**Pm**	**Fn**	**Cr**	**Ec**	**Pn**	**Cg**	**Co**	**Cs**	**Cc**	**Sm**	**Strepto spp**	**Av**	**Ao**	**Vp**	**Entero spp**	**Ef**	**Lacto spp.**	**S. mutans**
PLASMA	$1.0*10^9$	$1.3*10^3$	$4.2*10^6$	$1.5*10^5$	$2.4*10^5$	$1.2*10^7$	$5.7*10^6$	$8.9*10^6$	$1.4*10^6$	$7.1*10^6$	$1.2*10^2$	0	$3.9*10^5$	$2.6*10^5$	$3.2*10^2$	$1.2*10^5$	$8.8*10^5$	$2.9*10^3$	$3.3*10^5$	$1.9*10^5$	$1.9*10^4$	0	$1.0*10^5$	0
NON-PLASMA	$2.4*10^8$	0	$9.1*10^5$	$6.1*10^4$	$1.7*10^5$	$1.8*10^6$	$2.1*10^6$	$2*10^6$	$5*10^5$	$4.1*10^5$	$2.8*10^2$	$1.1*10^3$	$1.7*10^4$	$3.5*10^3$	$1.7*10^2$	$1.4*10^4$	$1.1*10^5$	$1.3*10^3$	$6.6*10^3$	$8.1*10^4$	$9.2*10^2$	0	$2.5*10^6$	$4*10^3$
p-value	0.059†	0.791	0.462	0.126	0.781	0.096	0.232	0.064 †	0.239	0.068†	1	0.794	0.864	0.143	0.905	**0.044†**	0.091	0.606	0.384	0.252	0.127	NA	**0.016** †	0.780