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Profile of bacteria colonizing the exposed bone of patients with anti-osteoclastic drug-related osteonecrosis of the jaws.

Running title: Microbiota of anti-osteoclastic drug osteonecrosis

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Abstract

Microbial etiology for anti-osteoclastic drug-related osteonecrosis of the jaw (ARONJ) was suggested. This study investigates any link between bacteria colonizing ARONJ sites and other oral cavity sites.

Microbiota samples of 10 ARONJ patients were collected from the exposed bone, adjacent teeth, contra-lateral teeth and tongue. DNA checkerboard hybridization was used for microbiota analysis with probes from human genomic DNA of 38 bacterial and 5 candida species guided by Socransky's bacterial complexes. Frequency and mean proportion of each bacterial species were used.

E. corrodens, *S. constellatus* and *F. nucleatum* were dominant in the ARONJ sites, and detected in most teeth samples. *S. aureus* was also dominant in ARONJ sites and tongue. Significant correlations were found between mean proportions of bacterial species colonizing adjacent teeth, contra-lateral teeth and tongue ($p < 0.001$, $R^2 > 0.69$). No significant correlation ($p > 0.05$, $R^2 < 0.025$) was found between bacteria colonizing ARONJ sites and other evaluated sites.

Within the study limitations, it was concluded that the primary sources of microorganisms colonizing ARONJ sites could be other sites such as teeth and tongue. The microbial profile of the necrotic bone is predominantly colonized by bacteria from Socransky's green and orange complexes, as well as with species associated with bone infections.

Key words: Anti-osteoclastic drug, jaw osteonecrosis, bacteria biofilm, DNA checkerboard, cancer.

Draft

Introduction

Bisphosphonates and receptor activator of nuclear factor kappa-B ligand (RANKL) inhibitors are drugs commonly used to inhibit bone resorption in the management of hematologic cancers like multiple myeloma, metastatic lesion from solid tumors such as breast and prostate, as well as in the treatment of metabolic bone disease like osteoporosis (Bundred 2012; Coleman and McCloskey 2011). Osteonecrosis of the jaw was recognized as a serious complication of anti-osteoclastic and anti-angiogenic drugs (MRONJ) (Ruggiero et al. 2014). Anti-osteoclastic drug-related osteonecrosis of the jaw (ARONJ) was defined as the presence of persistent, exposed, non-healing bone for more than 8 weeks in the maxillofacial region in patients treated with bisphosphonates or RANKL inhibitors but with no history of radiation to the head and neck region (Ruggiero et al. 2014). The etiology of ARONJ remains largely un-defined (Otto et al. 2010).

In a number of studies, bacterial etiology of ARONJ has been proposed (Hinson et al. 2014; Pushalkar et al. 2014; Sedghizadeh et al. 2012). The human oral cavity represents a reservoir of more than 700 bacterial species that colonize the teeth and mucosal surfaces of the mouth (Aas et al. 2005). Wei et al. used molecular techniques to characterize the microbiota associated with bisphosphonate osteonecrosis and identified *Streptococcus*, *Eubacterium* and *Pseudoramibacter* as the three top genera in these patients compared to *Parvimonas*, *Streptococcus* and *Fusobacterium* in control cancer patients with oral infection but with no history of bisphosphonate exposure (Wei et al. 2012). In another investigation, Ji et al. reported that oral antibiotics had limited effect on the bacterial population colonizing the sites of bone exposure in patients with bisphosphonate osteonecrosis (Ji et al. 2012). Although bacteria seem to play an

important role in the development of ARONJ, the source of bacterial infection is not clear. Therefore, the aim of this study was to determine if the profile of bacterial species colonizing ARONJ sites could be linked to the oral microbiota of other sites of patients with this condition.

Materials and methods

ARONJ patients

This study was approved by the McGill University Health Center Research Ethics Board. Patients diagnosed and treated for ARONJ at the Oral and Maxillofacial Clinic, Montreal General Hospital, were identified and signed an informed consent to participate in the study. The inclusion criteria were:

- 1- Male and/or female patients aged 18 years and older.
 - 2- Patients diagnosed and treated for bone exposure associated with ARONJ stage 1 or 2 between April and September 2012.
 - 3- Patients receiving oral and/or intravenous bisphosphonates or RANKL inhibitors.
- Patients with history of radiation to the head and neck area were excluded from the study. Details about patient age, gender, bisphosphonates, co-medications, periodontal health, main complaint and site of bone necrosis were documented.

Microbial sample collection

Microbial samples were collected using a microbrush (Microbrush International, Grafton, WI, USA) by rubbing it till saturation for 30 seconds. The use of microbrush was shown to be a suitable method for microbial biofilm collection (do Nascimento et al. 2009). Given possible inaccuracy in measuring the weight of the samples, we used standard-sized microbrush tips that

are capable to absorb a volume of about 6 μ L. To avoid any contamination, all microbrushes were sterilized and stored in sealed packs before use. At the time of sample collection, the microbrushes were uncovered, and direct collection of sample from the exposed necrotic bone, the supragingival part of the immediately adjacent teeth, the contra-lateral teeth and the dorsal surface of the tongue was obtained. Particular attention was paid to avoid any contact or contamination of the microbrushes with any other sites of the mouth. Immediately after collection, samples were individually disposed into microtubes containing 150 μ l of TE (10Mm Tris-HCl, 1Mm EDTA pH 7.6) and then 150 μ l of 0.5M sodium hydroxide to cause cell lysis and expose genetic material, according to protocol proposed by Socransky et al. (1994). Next, the microtubes were transported to the laboratory and stored at 4°C till DNA checkerboard hybridization. Sample collection did not cause any pain or discomfort to the patients and was performed without local anaesthesia.

DNA checkerboard hybridization

Bacterial and fungal DNA hybridization was performed using the checkerboard method as described previously (do Nascimento et al. 2010; Jabbour et al. 2013; Sunde et al. 2000). Hybridization probes were prepared by direct labeling (Amersham Gene Images AlkPhos Direct Labelling and Detection System, GE Healthcare, Buckinghamshire, UK) of genomic DNA from the 38 bacterial and 5 candida species listed in **Table 1**. Bacterial probe selection was based on relevance of the species to human oral health and guided by bacterial complexes described by Socransky et al. (Socransky et al. 1998). Suitable specificity and sensitivity of these probes were already tested and optimized for the detection of 10^4 cells (Socransky et al. 2004). Bacterial and fungal whole-genomic DNA extracted from biofilm samples from patients were denatured,

precipitated and blotted onto the hybridization membrane (Hybond N+, Amersham Biosciences, Buckinghamshire, UK) using MiniSlot 30 apparatus (Immunetics, MA, USA). As reference samples, defined amounts of genomic DNA corresponding to either 10^5 or 10^6 cells of each analyzed target species were mixed in a single tube, denatured, precipitated and applied on the membranes. Samples were fixed on the hybridization membrane using vacuum and baked for 2 hours at 80°C. Membranes were then pre-hybridized using an oven shaker (Amersham Biosciences) to control for temperature and humidity. Labeled probes were then introduced using a Miniblotter 45 device (Immunetics, Cambridge, MA, USA), and the membranes hybridized overnight at controlled temperature and humidity under gentle agitation. After washing, hybridization signals were detected by chemiluminescence using CDP-*Star* reagent (GE Healthcare), and membranes were exposed to ECL Hyperfilm-MP during 60 minutes (GE Healthcare). Hyperfilm images were digitized and quantified using TotalLab Quant analysis software (TotalLab Life Science Analysis Essentials, Newcastle upon Tyne, UK).

Statistical analyses

To adjust for any potential variation in the volume or weight of the samples, the mean proportion was used by calculating the percentage of each detected microbial species within each sample and then the percentages were averaged for each collection site using the formula below

$$\text{Mean proportions} = [(X_1/Y_1) + (X_2/Y_2) + (X_3/Y_3) \dots (X_Z/Y_Z)] / Z \times 100.$$

X= Detected signals from a species in a particular sample.

Y= Total detected signals from the corresponding sample.

Z= Number of samples: 10 for samples taken from tongue and sites of ARONJ, and 8 for samples taken from the teeth (2 edentate patients).

Multiple sites of exposed bone within the same patient were averaged and considered one sample. Spearman non-parametric correlation was used to assess the relationship between the proportions of bacterial species colonizing the exposed bone, adjacent teeth, contra-lateral teeth and tongue. Pairwise Wilcoxon signed rank test was used for within-group comparisons of mean bacterial proportions colonizing the adjacent and contra-lateral teeth. Mann–Whitney U test was used for comparisons of mean proportions of bacteria colonizing the sites of ARONJ and tongue between patients with or without dentures. All statistical analyses were performed using SPSS 19 with significance level $p < 0.05$.

Results

Patients' characteristics

ARONJ was noted clinically as exposed bone surrounded by inflamed soft tissues. A total of 10 ARONJ patients (male = 5, female = 5, mean age = 73.2 ± 10.8 years) were identified. Patients' characteristics are presented in **Table 2**. Exposure to bisphosphonates ranged from 1 to 8 years; subcutaneous denosumab and intravenous bisphosphonates were prescribed for 7 patients as adjuvant cancer medication, and oral bisphosphonates for 3 patients to treat osteoporosis; prednisone was the most frequent co-medication and was prescribed to 4 patients; seven patients had taken antibiotics in the 6 months prior to sample collection for systemic and/or oral infection control; only 2 patients were totally edentulous; multiple sites of bone exposure were noted in 3 patients.

Microbiological results

155 All probes hybridized with microbial whole genome extracted from the oral samples of ARONJ
156 patients. Most dominant bacteria in the site of exposed bone were *E. corrodens*, *S. gordonii*, *S.*
157 *constelatus*, *F. nucleatum*, *E. faecalis* and *S. aureus*. *E. corrodens* was the only bacterial species
158 detected in all sites of exposed bone (**Table 3**). The most dominant bacterial species colonizing
159 the teeth were *V. parvula*, *T. forsythia*, *S. gordonii*, *S. oralis*, *S. mitis* and *T. denticola*. The
160 tongue was mainly colonized by *V. parvula*, *S. mitis*, *S. gordonii*, *S. parasanguinis*, *S. oralis* and
161 *S. aureus* (**Table 3**). The mean proportions of bacteria per collection site are shown in **Figure 1**.

162
163 Significant correlations were found when assessing the relationships between the mean
164 proportions of the bacterial species colonizing the adjacent teeth, contra-lateral teeth and tongue.
165 However, no correlation was found between the mean proportions of bacterial species colonizing
166 the exposed bone and any other collection site in the mouth of ARONJ patients (**Figure 2**).

167
168 The mean proportion of *S. mutans* was significantly higher in the teeth adjacent to the exposed
169 bone compared to the contra-lateral teeth (**Figure 1**). Conversely, the mean proportions of *C.*
170 *krusei*, *K. pneumonia* and *N. mucosa* were significantly higher in the contra-lateral teeth when
171 compared to the teeth adjacent to the exposed bone. *E. faecalis* displayed the same tendency but
172 the difference did not reach statistical significance ($p=0.07$) (**Figure 1**).

173 Only few species showed statistically significant difference when comparing patients with or
174 without dentures. Significantly higher proportions of *P. micra* ($p=0.019$) and *T. denticola*
175 ($p=0.038$) was found in the sites of exposed bone in patients wearing dentures compared to
176 patients without dentures. *C. krusei* and *M. salivarium* had the same trend but did not reach
177 statistically significant difference ($p=0.067$). The proportions of *E. Faecalis* was significantly

higher ($p=0.038$), and the proportions of *T.denticola* tended to be significantly higher ($p=0.067$) in bacterial samples taken from the tongue of patients without dentures compared to patients with dentures

Discussion

Only few studies in the literature used molecular techniques to investigate the role of oral microbiota in the development of ARONJ (Ji et al., 2012; Wei et al., 2012). In the present investigation we employed the DNA checkerboard method using 43 genomic DNA probes prepared from human oral bacteria and fungi to assess any link between microorganisms colonizing the site of ARONJ and other sites of the oral cavity such as the teeth and the tongue.

Our study showed that the exposed bone exhibited a distinguishable bacterial profile from the other sites of the oral cavity. The remarkable presence of *E. corrodens*, in all sites of ARONJ, independently from its mean proportions, suggests a role of this species in the development of bone necrosis. This is consistent with previous reviews suggesting certain presence of *Eikenella* in the sites of bisphosphonates osteonecrosis (Hinson et al. 2014). *E. corrodens* is a Gram-negative facultative anaerobic bacillus and part of Socransky's green complex (Socransky et al. 1998). It has been identified in dentoalveolar abscess, osteoradionecrosis and it is possibility implicated in periodontal diseases (Kolenbrander et al. 2002; Store et al. 2005). In addition, the abundant presence of both *S. constellatus* and *F. nucleatum* was noted in the present investigation. These species are recognized as opportunistic pathogenic bacteria. They are part of Socransky's orange complex, and could be associated with infection of non-periodontal sites (Socransky et al. 1998; Store et al. 2005). Bacteria from the red complex (*T. forsythia*, *P. gingivalis* and *T. denticola*) were also found in our samples from exposed bone but in less

abundance. Species of this complex exhibit close relationship with species from the orange complex. They might not be able to colonize without the previous presence of bacteria from the orange complex (Socransky et al. 1998). In our cohort, patients wearing dentures seemed to have higher bacterial proportions from Socransky's red complex in the sites of exposed bone compared to patients without dentures.

In the present investigation, *E. corrodens*, *S. constellatus* and *F. nucleatum* were also found in almost all the samples collect from the adjacent and contra-lateral teeth, and detected in lower abundance in samples taken from the tongue. This suggests that bacteria from green and orange complexes colonizing the teeth and tongue of ARONJ patients might be synergistically implicated in the development of necrosis.

Furthermore, *S. aureus* was the 6th most dominant species in the site of exposed bone and had high proportions in samples taken from the tongue. The remarkable presence of *S. aureus* in bone samples could be explained by this species' ability to adhere to bone by means of its collagen receptors (Holderbaum et al. 1987). Its high incidence in samples taken from the exposed bone could also be related to an enhanced adhesion promoted by bisphosphonates (Kobayashi et al. 2010; Kos et al. 2013).

A particular feature of bone infections by *S. aureus* and *E. corrodens* species is the destruction of mineralized tissue resulting from the ability of *S. aureus* protein A to activate nuclear factor kappa B, which in turn activates osteoclastic activity in the area of infection (Claro et al. 2013). *E. corrodens* also produces a surface-associated material that stimulates the synthesis of Tumor Necrosis Factor alpha (TNF α) leading to potent bone resorption (Meghji et al. 1995; Meghji et al. 1994). Although bone resorption was not measured clinically or radiographically in the

current investigation, it was not visually apparent in our cohort of patients. It is known that bisphosphonates and RANKL inhibitors decreases osteoclastic activity and reduce bone resorption. Therefore, the absence of bone resorption in the area of necrotic bone could be the direct result of bisphosphonates and RANKL inhibitors administration (Jabbour et al. 2016).

The results of the present study showed rare presence of *A. actinomycetemcomitans* in the sites of ARONJ. This observation is consistent with Wei et al. who reported low incidence of *A. actinomycetemcomitans* in their bone samples using molecular techniques (Wei et al. 2012). However, considerable presence of *A. actinomycetemcomitans* was previously observed using histological sections (Hansen et al. 2007; Hansen et al. 2006; Hinson et al. 2014). Although *A. actinomycetemcomitans* has often been associated with periodontal diseases (Amano 2010; Henderson et al. 2010; Slots and Ting 1999), its sporadic presence in the area of exposed bone in our samples suggests a minor role of this species in the development of ARONJ.

In the current investigation, significant correlations were found between the mean bacterial proportions colonizing the adjacent teeth, contra-lateral teeth and tongue. These correlations suggest that the prescribed medications had similar impact on the bacteria colonizing the surface of vital structures such as teeth and tongue, but not the surface of necrotic exposed bone. Differences noted in the proportions of species colonizing the exposed bone, tongue and the supragingival dental biofilm could be attributed to the distinct surface characteristics of each, and the inherent ability of specific microbial species to selectively colonize them. In the present study, we included samples from the tongue since previous reports showed that the dorsum of the tongue could house an organized biofilm and act as reservoir for the re-colonization of oral surfaces by pathogenic species (Faveri et al. 2006; Mager et al. 2003).

Differences in the mean proportions of *C. krusei*, *E. faecalis*, *K. pneumonia*, *N. mucosa* and *S. mutans* colonizing the adjacent and contra-lateral teeth, although significant, were small (**Figure 1**). In addition, there was a similar frequency of these species in the adjacent and contra-lateral teeth (**Table 3**), suggesting that they might not be directly related to the development of ARONJ.

In future studies, a longitudinal design could be useful to further investigate the link between bacterial complexes and development of ARONJ. Although the cross-sectional design of the current investigation provided information on the microbial species associated with ARONJ, it does not provide information about the sequence of events that led to ARONJ and whether ARONJ is the result of microbial infection with bacteria colonizing the teeth and tongue. Larger number of patients is desired to better understand this link and whether other factors such as medications, gender or ethnicity could play a role in the development of ARONJ.

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Conflict of Interest

None.

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Figure 1: Mean proportions ($\% \pm \text{SEM}$) of the microbial species detected. Statistically significant difference between the mean bacterial species colonizing the adjacent and contra-lateral teeth (Pairwise Wilcoxon signed rank test $*p \leq 0.05$, $**p < 0.01$, $^{\dagger}p = 0.07$).

Figure 2: Scatter plots of the mean bacterial proportions (%) showing significant correlations between the bacteria colonizing the adjacent teeth, contra-lateral teeth and tongue. No correlation was found between the bacteria colonizing the exposed bone and other evaluated sites of the mouth.

Table 1: Human DNA microbial species used to prepare probes for cross-reaction with species extracted from the oral cavity of humans.

Species	Reference
<i>Aggregatibacter actinomycetemcomitans a</i>	ATCC 29523
<i>Aggregatibacter actinomycetemcomitans b</i>	ATCC 29522
<i>Bacteroides fragilis</i>	ATCC 25285
<i>Campylobacter rectus</i>	ATCC 33238
<i>Candida albicans</i>	ATCC 10231
<i>Candida dubliniensis</i>	ATCC MYA 646
<i>Candida glabrata</i>	ATCC 90030
<i>Candida krusei</i>	ATCC 6258
<i>Candida tropicalis</i>	ATCC 750
<i>Capnocytophaga gingivalis</i>	ATCC 33624
<i>Eikenella corrodens</i>	ATCC 23834
<i>Enterococcus faecalis</i>	ATCC 51299
<i>Escherichia coli</i>	ATCC 10798
<i>Fusobacterium nucleatum</i>	ATCC 25586
<i>Fusobacterium periodonticum</i>	ATCC 33693
<i>Klebsiella pneumoniae</i>	ATCC 700721
<i>Lactobacillus casei</i>	ATCC 393
<i>Mycoplasma salivarium</i>	ATCC 23064
<i>Neisseria mucosa</i>	ATCC 25996
<i>Parvimonas micra</i>	ATCC 33270
<i>Peptostreptococcus anaerobius</i>	ATCC 49031
<i>Porphyromonas endodontalis</i>	ATCC 35406
<i>Porphyromonas gingivalis</i>	ATCC 33277
<i>Prevotella intermedia</i>	ATCC 25611
<i>Prevotella melaninogenica</i>	ATCC 25845
<i>Prevotella nigrescens</i>	ATCC 33563
<i>Pseudomonas aeruginosa</i>	ATCC 27853
<i>Pseudomonas putida</i>	ATCC 12633
<i>Solobacterium moorei</i>	CCUG 39336
<i>Staphylococcus aureus</i>	ATCC 25923
<i>Staphylococcus pasteurii</i>	ATCC 51129
<i>Streptococcus constellatus</i>	ATCC 27823
<i>Streptococcus gordonii</i>	ATCC 10558
<i>Streptococcus mitis</i>	ATCC 49456
<i>Streptococcus mutans</i>	ATCC 25175
<i>Streptococcus oralis</i>	ATCC 35037
<i>Streptococcus parasanguinis</i>	ATCC 15911
<i>Streptococcus salivarius</i>	ATCC 25975
<i>Streptococcus sanguinis</i>	ATCC 10556
<i>Streptococcus sobrinus</i>	ATCC 27352
<i>Tannerella forsythia</i>	ATCC 43037
<i>Treponema denticola</i>	ATCC 35405
<i>Veillonella parvula</i>	ATCC 10790

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anti-osteoclastic drug	anti-osteoclastic drug stopped	Co-medications (last 6 months)	Antibiotics (last 6 months)	Site of ARONJ (stage)	Plaque (adjacent teeth)	Plaque (Contra-lateral teeth)	Presence of denture
Zoledronic acid (IV)	No	Prednisone Abiraterone Sunitinib	Amoxicillin + clavulanic acid Moxifloxacin	44, 43 lingual ridge (II)	3	3	Partial
Zoledronic acid (IV)	Yes	Sunitinib	-	37, 38 (I) 47 (I)	-	-	Complete
Zoledronic acid (IV)	Yes	Prednisone	Clindamycin	37, 38 lingual ridge (I)	2	1	Partial
Risedronate (PO)	No	Prednisone	Moxifloxacin	46 (II)	1	1	-
Alendronate (PO)	Yes	-	Amoxicillin	37 (I)	1	1	-
Denosumab (SC)	No	Prednisone	-	36 (I) 15 (I)	1 1	1 1	-
Alendronate (PO)	No	-	Penicillin VK	47, 46, 45, 44 (II)	-	-	Complete
Pamidronate (IV)	No	Sunitinib	Teva-amoxicillin Metranidazole	12 buccal ridge (I) 23, 24, 25 (I) 47 (II)	3 3 2	3 3 2	-
Alendronate (PO) Pamidronate (IV)	No	Tamoxifen, Aramasin	-	36, 37 lingual ridge (I)	1	1	-
Zoledronic acid (IV)	Yes	Fulvestrant,	Amoxicillin+ clavilacule acid Ciprofloxacin	47, 48 (II)	3	1	-

ed on the gingival third with explorer, 2 = Plaque is visible, 3 = Tooth surface covered with abundant plaqu

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Table 3: Bacterial species per collection site and their detected frequencies ordered by decreasing mean proportions illustrated in **Figure 1**.

Exposed bone (Number of sites=10)	Adjacent teeth (Number of sites =8)	Contra-lateral teeth (Number of sites =8)	Tongue (Number of sites =10)
<i>E.corrodens</i> (10)	<i>V.parvula</i> (8)	<i>V.parvula</i> (8)	<i>V.parvula</i> (10)
<i>S.gordonii</i> (8)	<i>T.forsythia</i> (8)	<i>S.gordonii</i> (8)	<i>S.mitis</i> (10)
<i>S.constelatus</i> (8)	<i>S.gordonii</i> (8)	<i>S.oralis</i> (8)	<i>S.gordonii</i> (8)
<i>F.nucleatum</i> (8)	<i>S.mitis</i> (8)	<i>T.denticola</i> (7)	<i>S.parasanguinis</i> (10)
<i>E.faecalis</i> (9)	<i>S.oralis</i> (8)	<i>T.forsythia</i> (8)	<i>S.oralis</i> (10)
<i>S.aureus</i> (8)	<i>S.mutans</i> (8)	<i>S.mitis</i> (8)	<i>S.aureus</i> (10)
<i>S.mitis</i> (8)	<i>S.sanguinis</i> (8)	<i>S.sanguinis</i> (8)	<i>P.intermedia</i> (7)
<i>N.mucosa</i> (9)	<i>Aa_a</i> (8)	<i>P.intermedia</i> (6)	<i>S.mutans</i> (10)
<i>T.forsythia</i> (9)	<i>S.sobrinus</i> (8)	<i>S.mutans</i> (8)	<i>S.sanguinis</i> (10)
<i>E.coli</i> (6)	<i>T.denticola</i> (6)	<i>P.melaninogenica</i> (7)	<i>E.corrodens</i> (9)
<i>S.sobrinus</i> (8)	<i>P.gingivalis</i> (7)	<i>C.gingivalis</i> (7)	<i>Aa_a</i> (10)
<i>P.gingivalis</i> (7)	<i>S.parasanguinis</i> (8)	<i>F.nucleatum</i> (8)	<i>P.gingivalis</i> (7)
<i>P.aeruginosa</i> (8)	<i>P.melaninogenica</i> (6)	<i>S.pasteuri</i> (8)	<i>P.melaninogenica</i> (7)
<i>K.pneumoniae</i> (7)	<i>S.constelatus</i> (8)	<i>P.micra</i> (8)	<i>T.forsythia</i> (10)
<i>S.parasanguinis</i> (5)	<i>F.nucleatum</i> (7)	<i>S.parasanguinis</i> (8)	<i>S.sobrinus</i> (10)
<i>P.endodontalis</i> (8)	<i>S.pasteuri</i> (7)	<i>Aa_a</i> (8)	<i>S.pasteuri</i> (9)
<i>P.intermedia</i> (6)	<i>S.salivarius</i> (8)	<i>S.aureus</i> (8)	<i>M.salivarium</i> (10)
<i>P.anaerobios</i> (8)	<i>S.aureus</i> (7)	<i>S.sobrinus</i> (8)	<i>C.gingivalis</i> (8)
<i>S.moorei</i> (6)	<i>C.gingivalis</i> (6)	<i>P.gingivalis</i> (8)	<i>Aa_b</i> (7)
<i>C.krusei</i> (7)	<i>P.nigrescens</i> (7)	<i>P.nigrescens</i> (8)	<i>L.casei</i> (10)
<i>F.periodonticum</i> (7)	<i>P.intermedia</i> (4)	<i>E.corrodens</i> (8)	<i>T.denticola</i> (7)
<i>M.salivarium</i> (7)	<i>C.albicans</i> (6)	<i>S.moorei</i> (8)	<i>P.nigrescens</i> (7)
<i>S.pasteuri</i> (6)	<i>P.putida</i> (8)	<i>C.rectus</i> (7)	<i>P.putida</i> (8)
<i>C.glabrata</i> (7)	<i>P.micra</i> (6)	<i>F.periodonticum</i> (8)	<i>S.moorei</i> (8)
<i>C.albicans</i> (7)	<i>L.casei</i> (7)	<i>S.salivarius</i> (8)	<i>C.rectus</i> (6)
<i>C.dubliniensis</i> (2)	<i>S.moorei</i> (7)	<i>L.casei</i> (8)	<i>F.nucleatum</i> (7)
<i>S.oralis</i> (6)	<i>C.rectus</i> (5)	<i>P.putida</i> (8)	<i>E.faecalis</i> (9)
<i>P.melaninogenica</i> (6)	<i>M.salivarium</i> (7)	<i>M.salivarium</i> (8)	<i>K.pneumoniae</i> (8)
<i>P.micra</i> (7)	<i>C.glabrata</i> (7)	<i>S.constelatus</i> (8)	<i>P.micra</i> (7)
<i>L.casei</i> (6)	<i>F.periodonticum</i> (6)	<i>P.anaerobios</i> (8)	<i>S.salivarius</i> (7)
<i>P.putida</i> (5)	<i>E.corrodens</i> (7)	<i>K.pneumoniae</i> (8)	<i>N.mucosa</i> (4)
<i>S.salivarius</i> (6)	<i>P.anaerobios</i> (7)	<i>B.fragilis</i> (6)	<i>C.albicans</i> (4)
<i>C.tropicalis</i> (5)	<i>P.endodontalis</i> (7)	<i>C.glabrata</i> (8)	<i>S.constelatus</i> (2)
<i>S.sanguinis</i> (4)	<i>E.coli</i> (6)	<i>E.faecalis</i> (8)	<i>E.coli</i> (3)
<i>V.parvula</i> (4)	<i>K.pneumoniae</i> (7)	<i>Aa_b</i> (6)	<i>P.endodontalis</i> (3)
<i>T.denticola</i> (5)	<i>B.fragilis</i> (5)	<i>P.endodontalis</i> (8)	<i>P.anaerobios</i> (3)
<i>S.mutans</i> (4)	<i>C.dubliniensis</i> (6)	<i>N.mucosa</i> (8)	<i>B.fragilis</i> (2)
<i>P.nigrescens</i> (5)	<i>E.faecalis</i> (7)	<i>E.coli</i> (6)	<i>F.periodonticum</i> (1)
<i>Aa_a</i> (1)	<i>Aa_b</i> (4)	<i>C.albicans</i> (6)	<i>P.aeruginosa</i> (1)
<i>C.gingivalis</i> (0)	<i>N.mucosa</i> (7)	<i>P.aeruginosa</i> (8)	<i>C.tropicalis</i> (1)
<i>Aa_b</i> (0)	<i>P.aeruginosa</i> (6)	<i>C.tropicalis</i> (8)	<i>C.glabrata</i> (0)

C.rectus (0)
B.fragilis (0)

C.tropicalis (6)
C.krusei (6)

C.dubliniensis (6)
C.krusei (6)

C.dubliniensis (0)
C.krusei (0)

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Figure 1: Mean proportions (% ± SEM) of the microbial species detected. Statistically significant difference between the mean bacterial species colonizing the adjacent and contra-lateral teeth (Pairwise Wilcoxon signed rank test *p ≤0.05, **p<0.01, †p=0.07)
750x1422mm (96 x 96 DPI)

Figure 2: Scatter plots of the mean bacterial proportions (%) showing significant correlations between the bacteria colonizing the adjacent teeth, contra-lateral teeth and tongue. No correlation was found between the bacteria colonizing the exposed bone and other evaluated sites of the mouth.

1270x952mm (61 x 61 DPI)