Changes of Microbial Community in Treated Peri-implantitis Sites: An Experimental Study in Beagle Dogs

Yu Wei WU1*, Hui ZHENG2,3*, Xue Fen LI4, Hui LU1, Song He LU1, Xu Hong LIU1, Qiang ZENG3, Feng CHEN4, Jiu Xiang LIN3, Zhi Hui TANG1

Objective: To investigate the changes of the bacterial community in the oral environment of beagle dogs to gain insights on the possible causes of failed therapy in peri-implantitis.

Methods: Beagles were used as models for experimental peri-implantitis. Samples from peri-implant soft tissue (supra- and submarginal) ligature, and contaminated surface of peri-implantitis sites were collected and analyzed by sequencing the bacterial 16S rRNA gene.

Results: The residual microbial community from the curettes-treated implant surface contained a variety of microorganisms, including periodontal pathogens, which showed no changes in their composition and structure.

Conclusion: It is possible that the residual bacterial community remained unchanged and this was the cause of recurrent episodes of inflammation.

Key words: peri-implantitis, microbial communities, peri-implant mucositis, peri-implant soft tissue, biofilm.


Dental implants are the most convenient and comfortable way to replace missing teeth1. Peri-implantitis is caused by long-term exposure to oral microbial pathogens that are difficult to eradicate, and is one of the most important reasons for failure of the dental implant treatment2. Due to the experience gained from periodontitis treatment in recent decades, the therapy for peri-implantitis currently comprises two phases: nonsurgical and surgical. The nonsurgical phase consists of the debridement by mechanical, ultrasonic, or laser devices3,4, either alone or combined with antiseptic and/or antibiotic agents3,5,6, while the surgical phase comprises resective or regenerative techniques7,8. It is widely known that periodontitis treatment, which aims to control the bacterial infection around the periodontal tissue, has a reasonable predictability of success; however, despite the various treatment modalities for peri-implantitis, it is difficult to control the soft tissue infection and prevent the loss of hard tissues around the implant9,10. It has been observed that peri-implantitis does not respond to traditional nonsurgical treatment11. Leonhardt et al12 implemented a strategy for the surgical exposure of lesions, cleaning of the implants, and antimicrobial treatment for advanced peri-implantitis, and reported a success rate of only 58% during a 5-year follow-up period. A retrospective study reported that 37 implants (6% of included implants) had to be removed in 34 patients with a history of peri-implantitis surgery13. The numbers of pathogens must be successfully reduced to prevent the development of peri-implantitis. Thus, to re-establish the dynamic balance between the host and the microbiome is of utmost importance14; however, the
various treatment modalities for peri-implantitis affect the entire oral cavity and do not differentiate between implant surface and tooth surface/structure\textsuperscript{15,16}.

Therefore, we hypothesised whether our curettage treatment of peri-implantitis could achieve the goal of changing the bacterial community without affecting the host. To prove this, the microbial community in the oral cavity of beagles was compared before and after surgical debridement of peri-implantitis by using 16S rRNA gene sequencing, which was used for the first time to analyse peri-implantitis-associated pathogens.

**Materials and methods**

Beagle dogs were used to conduct the peri-implantitis model; the oral bacterial communities were collected from beagles at different sites and different depths, as well as the ligature and infected surfaces. Changes in the microbial community were compared before and after the peri-implantitis treatment. The ethical approval for this study was obtained from the Institutional Review, Laboratory of Animal Welfare, Ethic Branch Board at the Peking University (approval no. LA2015211).

**Establishment of the animal model for peri-implantitis**

Five healthy male 12-month-old beagles, weighing 15 to 20 kg were used. The dogs were given the same soft diets. General anaesthesia was administered with sodium pentobarbital (30 mg/kg iv) during all surgical procedures. In addition, adequate measures were taken to minimise pain or discomfort for all animals.

Three months after tooth extraction, 20 implants (Straumann Bone-Level SLActive Dental Implant System, Basel, Switzerland), which were 8 mm x 3.3 mm with uniform thread design, were placed in the beagle dogs. Three months after the implant placement, healing abutments were delivered, and cotton floss ligatures were placed around the implant neck (Fig 1a, c, and e). The ligature position was adjusted monthly to facilitate plaque accumulation (Fig 1b). Five months later, the ligatures were removed following intraoral periapical radiological examination to confirm the supporting bone loss (Fig 1d and 1f).

**Sample collecting and processing**

When the dogs were sedated, the ligatures (n = 5) were removed. Each supramargin sample (n = 18) was collected from the teeth surface before conducting surgery using a curette, and then pooled and re-suspended in 1 ml phosphate buffered saline (PBS) solution. Subsequently, the peri-implantitis submargin plaque (n = 18) was taken from the deepest pockets using a sterile scaler. Bacterial samples were placed in a 1-ml eppendorf tube containing 200 μl sterile tris(hydroxymethyl)aminomethane- ethylenediaminetetraacetic acid (Tris-EDTA) (TE) buffer solution. The tube was then temporarily stored on ice. After flap elevation by the same dentist and removal of calculus and granulation tissue using Gracey curettes,
the chipping fragments of the titanium surfaces (n = 13) were cleaned with a R-brush polishing tool (Neobiotech, Guro-gu, Seoul, Korea), and then collected using a 50-ml sterilised syringe, which was suspended in normal saline solution. A regular sized R-Brush was connected to a contra-angle head of 1:1 and centrifuged at 8,000 rpm under copious normal saline irrigation. Each thread was washed for 30 to 60 seconds and contaminated threads were washed for nearly 5 minutes. It is known that the R-Brush can alter the original soft surface and produce a new, rougher surface; which is not a polished-like surface but is, instead, a rough-like surface. All microbial samples infected with the polymicrobial consortium were centrifuged at 12,000 \( \times \) g for 10 min at 4°C, and the precipitate was discarded. The samples were transported in an ice box and stored at -80°C.

**DNA extraction and PCR amplification**

The bacterial microbiome DNA was extracted using the QIAamp DNA Microbiome Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Genomic DNA from the ligatures and titanium fragments was extracted using a TIANamp swab DNA kit (Qiagen). The primers 338F (5’-ACTCCTACGGGAGGCAGCAG-3’) and 806R (5’-GACTACHVGGGTWTCTAAT-3’), spanning the V3-V4 hypervariable region of the bacterial 16S rRNA gene sequence, were used for PCR amplification and barcoded pyrosequencing; the latter technique was performed using the MiSeq platform 2 x 300 bp paired-end sequencing system (Illumina, San Diego, CA, USA). The sequences obtained were submitted to the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) (SRP043555).

**Data processing and statistical analysis of the 16S rRNA gene sequence**

Sequence visualisation and statistical analyses were performed with FASTQC (http://www.bioinformatics. babraham.ac.uk/projects/fastqc/). The Sickle software (https://github.com/najoshi/sickle) was used to retrieve the paired sequences with quality scores above a threshold of 25 and a length greater than 30 bp. The paired-end reads were merged with FLASH (http://ccb.jhu. edu/software/FLASH), and the reads with output length below 450 were filtered\(^{17}\).

The 16S rRNA gene sequences were quality-checked via clustering the amplicon sequence reads into operational taxonomic units (OTUs), within 0.03 difference (corresponding to 97% similarity) using the QIIME UCLUST software; sequences were compared against sequences in the Greengenes bacterial 16S rRNA database as previously described\(^{18}\). Low abundance phyla were omitted. Chao1 and Shannon indices were used to estimate the richness and evenness related to the number of observed OTUs. Phylogenetic distances and UniFrac distances were also calculated.

The Alpha diversity indices were calculated using R (version 3.13). The Student’s t-test was used to compare the alpha diversities. Beta differences were analysed using multivariate ANOVA (Adonis) of the UniFrac distance metric, implemented in the vegan function of the R package. For further analysis, UniFrac distances were analysed via a principal coordinates analysis (PCoA). Relative richness before and after treatment were compared using the Wilcoxon test. To build the bacterial network, the Spearman’s rank test was used if both the correlation coefficient (\( r \geq 0.5 \)) and the statistical differences were significant (\( P < 0.05 \)). \( P \) values were not corrected for multiple comparisons.

**Results**

**Establishment of the animal model for peri-implantitis**

Three months after the implant osteointegration (Fig 1a, c, and e), cotton floss ligatures were placed around the implant neck (Fig 1b, d, and f). Five months after the ligature, the experimental animal model for peri-implantitis was successfully established and confirmed by clinical examinations. An inflammatory process developed in the soft tissues, including bleeding on probing and suppuration. Radiographic imaging demonstrated a crater-shaped circumferential bony defect around the implant (Fig 1d), showing bone loss around the implant. The marginal bone level was located along the central or apical third of the intraosseous part of the implant (Fig 1d and f).

**Bacterial composition of peri-implantitis in the beagle models**

To further understand the possible reasons why some peri-implantitis patients do not gain fully recovery, the etiology of the microbial community at different sites (such as, supramargin, submargin, ligature, and contaminated surfaces) around peri-implantitis (Fig 1f) was thoroughly investigated. It was found that bacteria belonging to the phyla Synergistetes (\([25.7 \pm 12.4\%]\)), Proteobacteria (\([25.7 \pm 12.7\%]\)), Firmicutes (\([18.3 \pm 9.4\%]\)), TM7 (\([7.0 \pm 5.2\%]\)), Fusobacteria (\([6.9 \pm 5.0\%]\), and
Actinobacteria ([5.3 ± 4.0]%) were the most represented microbial communities in the supramargin samples, with Synergistetes being the most abundant among these. On the other hand, Synergistetes ([32.6 ± 11.0]%), Firmicutes ([30.4 ± 11.3]%), Bacteroidetes ([13.1 ± 11.1]%), Proteobacteria ([6.1 ± 4.0]%), and Fusobacteria ([6.0 ± 8.9]%) were the most abundant phyla observed in the submargin site samples.

Comparison of the microbial community structure between different sites of the beagle peri-implantitis

The PCoA analysis based on the unweighted UniFrac distance matrices showed that the samples from supramargin, submargin, ligature and implant surface were clustered together separately, and different sampling sites were separated from each other, indicating that the microbial composition of different peri-implant parts of the beagle dogs were significantly different.

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The PCoA analysis based on the unweighted UniFrac distance matrices showed distinct departures and the space distance in the co-ordinates indicated the extent of similarity among the submargin group, supramargin group, ligature group and the implant surface group (Fig 2). Furthermore, the surface group showed longer distance compared to others, indicating that the community characteristics of implant surface microorganisms were significantly different from the supramargin, submargin and ligature groups (Fig 2, Adonis, $P < 0.05$). The difference between the remaining flora on the implant surface and the other three groups was also

**Fig 2** The principal coordinates analysis (PCoA) showing bacterial differences at different parts of the implants in the beagle dogs. PCoA analysis based on unweighted UniFrac distance matrices showed that the samples from supramargin, submargin, ligature and implant surface were clustered together separately, and different sampling sites were separated from each other, indicating that the microbial composition of different peri-implant parts of the beagle dogs were significantly different.

**Fig 3** Comparison of alpha diversity of microbial communities in different parts of peri-implantitis in beagle dogs. 15,000 sequences were randomly selected from each sample to calculate the alpha diversity of the microbial community using independent sample t test (NS showed no significant difference, *$P < 0.05$*, **$P < 0.01$**). (a) The observed operational taxonomic units (OTUs) represent the types of OTUs detected in the sample; (b) The Chao1 index indicates the predicted OTU species in the population; (c) The Shannon index was the diversity index of the microbial community; (d) The phylogenetic distance was considered as the microorganism community diversity index.
observed in the diversity of Alpha, and the abundance and diversity of the flora, which was significantly lower than those of the other three groups (Fig 3).

**The remaining bacterial composition after surgical flap and mechanical debridement**

The remaining bacterial composition after surgical flap and mechanical debridement was assessed and compared with sequences deposited in the Greengenes 16S rRNA gene database, via amplifying the V3-V4 region of the 16S rRNA gene from the five samples (Fig 4). *Synergistetes* remained to be the most abundant phylum among the three samples, accounting for more than half of all species (53.0% to 84.0%). In contrast, *Synergistetes* comprised less than 0.1% in the other two samples, and TM7 remained the most abundant (73.6% and 13.5%). *Firmicutes* were abundant in all the samples (14.4 ± 12.2%). A total of 48 genera were examined in the surface of the treated implant, with 40 genera being observed in the submargin group, and 33 genera were shared by both groups. Fifteen genera remained specific to the implant surface group and 7 genera to the submargin group (Fig 5). Several other taxons were found on the surface of the treated implant of the submargin group, demonstrating how the bacterial community at the contaminated implant surface may be complex after the mechanical debridement.

**Periodontal bacterial pathogens in the sample remaining from the surface fragments of the implant**

A microbial copolymer network together with submargin flora and surface residual flora was constructed to explore the microbial nexus (Fig 6). Each node represents a genus. A total of 135 correlations were drawn by 36 nodes and the final network showed that the majority of the correlations were positive (n = 127). All genera presented in the network were detected in the submargin group (n = 36), and most of them detected in the implant surface group (n = 31), suggesting a close correlation between the microbial composition of the submargin and surface groups. Furthermore, almost all known putative periodontal pathogens, such as *Porphyromonas*, *Tannerella*, *Treponema*, *Prevotella* were found in the implant surface fragments after surgery (Fig 6).

**Discussion**

Peri-implantitis is considered to be an inflammatory disease resulting from the interaction between oral pathogens and the host response. The main treatment consists of removing the local oral biofilm. Therefore, we aimed to assess changes in oral microbial communities before and after treatment of induced-peri-implantitis in beagle dogs. As previously mentioned, the microbial composition and structure of oral plaque in peri-implantitis was investigated at different sites by using an animal model. Unlike the tooth root, the implant has a more complex structure and much coarser surface, making the removal of the bacterial biofilm more difficult. We assumed that, after treatment, the remaining bacteria on the surface and around the implant affected the curative effect.
Many instruments, such as plastic and metal curettes, ultrasonic scalers, rubber polish cups, are used for removing the gingival and submargin plaque biofilm around the implant, and are mainly designed to maintain a relatively smooth tooth surface, removal of dental calculus and plaque; however, these tools do not achieve a satisfying result when used on rough surface debridement\textsuperscript{21}. In addition, the direct contact of metal scaler and the implant surface may damage the surface structure, which promotes further bacterial colonisation on the surface\textsuperscript{22}. Persson et al\textsuperscript{23} and Renvert et al\textsuperscript{24} observed that the debridement of peri-implantitis could only partially reduce the amount of the submargin plaque and improve the clinical indexes. Schwarz et al\textsuperscript{25} reported that conventional mechanical methods such as the periodontal curettes and ultrasonic scalers could not thoroughly remove the plaque biofilm from the implant surface, and that 30\% to 40\% of biofilm remained intact after ultrasound scaling. Charalampakis et al\textsuperscript{26} detected specific oral microbes by using selective medium and found that the microbiological detection rate on the implant surface after surgery was about 50\%. In the present study, the submargin plaque on the surface of the canine implant was collected after the flap surgery and using a titanium rotary brush (R-brush, Neobiotech) (Fig 1g and h). PCR was used to amplify fragments of the V3-V4 variable region of the 16S rRNA gene to assess whether microorganisms were present. From a total of 13 samples, microorganisms were detected in five only. The low detection rate may be due to the loss of samples occurring during the sampling process (cooling water was poured on the infected implant surface during mechanical debridement and thus, some bacteria were lost during drainage), or during genomic DNA extraction, or even possibly at a later stage of the protocol. In fact, the residual rate of bacteria may be greater than the one observed here. Furthermore, residual microorganisms were detected on the implant surface after the flap surgery and mechanical debridement; this indicates that the periodontal treatment and mechanical debridement are inadequate techniques for biofilm removal from implants. Removal techniques targeting plaque, such as implantoplasty, specific to treat the implant surface, are required\textsuperscript{27}.

In the present study, a greater variety of microorganisms was detected in the remaining flora of the...
implant surface compared with the submargin flora group (Figs 2 to 6). It was also found that the microbial copolymerisation pattern occurred on the submargin and surface of the implant (Fig 2), and the interaction between these microorganisms remained a key factor in the resilience of the flora. Due to the presence of residual microorganisms after treatment and the close association between them, the submargin flora of the implant surface after discontinuation of intervention can quickly return to the pre-treatment state\(^{28}\). In addition, in the present study the periodontal pathogens belonging to the network node occupied a large proportion of the residual microbial symbiosis network in the submargin implant surface (Figs 3 to 5). Among the periodontal bacteria and other oral microbes detected, a close positive correlation was found (Fig 6), suggesting that the microbial profile almost remained the same after treatment. Hulting et al\(^{29}\) and Rams et al\(^{30}\) found that periodontal pathogens (such as Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia and Treponema denticola) were frequently detected at the lesion site around the implant. Our results suggest that periodontal pathogens form a cohesive network in the submargin site of infected implants, indicating that periodontal pathogens are not only an important factor in the development of disease around the implants, but are also an important factor in the treatment failure of peri-implantitis. This was in line with the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions\(^{2,31}\). In addition to periodontal pathogens, members of the genus Fusarium are closely associated with other microorganisms forming microbial cohesive networks\(^{32,33}\). Fusobacterium (in particular Fusobacterium nucleatum) has the ability to adhere to a variety of other oral microbes and may play an important role in the recovery of the flora after treatment\(^{32}\). It has been shown that antimicrobial photodynamic therapy (aPDT) and the topical application of tetracycline successfully decontaminated the infected implant surfaces\(^{33}\).

Although the findings presented here suggested that the treatment failure of peri-implantitis was inseparable from the residual microbes on the implant surface, it cannot be ruled out the possibility of bacterial contamination from other sites (such as submargin bacteria from adjacent teeth) to the lesion area. Some studies have shown that periodontal pathogens can disperse from the remaining natural teeth to the implant site\(^{34,35}\). Papaioannou et al\(^{34}\) found a similar submargin microbial composition with the same depth in the periodontal pocket and around the implant in the same patient\(^{34}\). It has also been shown that peri-implant submargin microbes derived from adjacent teeth can constitute a great risk of infection around the implant in patients with a history of periodontitis\(^{36}\). Karoussis et al\(^{37}\) found that the history of chronic periodontitis and the increased probing depth around the implant, the marginal bone loss and the incidence of peri-implantitis were significantly correlated. Quirynen et al\(^{38}\) pointed out that the incidence of long-term implant loss and alveolar bone resorption in periodontitis patients was greater in patients with rough surface implants and in cases where initial periodontal therapy had not been performed. These results suggest that periodontal pathogens from adjacent teeth are also important etiologies to take into account in the development of diseases around implants, but do not account for submargin microorganisms after treatment of implant-related diseases from adjacent teeth.

A multicenter, large-scale retrospective trial found that the results of peri-implantitis treatment were not associated with factors such as patient’s oral hygiene status, history and presence of periodontitis\(^{26}\). In a systematic review by Schou et al\(^{39}\), the history of periodontitis showed no significant effect on the retention rate of the implant. However, the incidence of peri-implant disease in patients with periodontal disease and bone loss around the implant increased significantly. In summary, the periodontal pathogens from adjacent teeth with periodontitis are important causes of peri-implant diseases. Nevertheless, once the disease has developed into peri-implantitis due to the complex structure of the implant and its rough surface, a stable microbial ecosystem at the implant submargin sites is formed, making it difficult to remove it completely. Treatment failure may be due to residual submargin bacteria on the implant surface after treatment, rather than from the adjacent teeth.

Beagle dogs are often used as periodontitis or peri-implantitis animal models, and peri-implant tissue destruction from ligation of silk induced plaque accumulation is the most commonly used method\(^{40-42}\). Due to a variety of species present in the oral cavity, the degree of similarities (or differences) of the flora around the implant between humans and dogs is one of the main concern for researchers using this animal model. Dahlén et al\(^{40}\) found that the peri-implant submargin flora in humans and dogs were similar at the genus level, but were significantly different at the species level. The 16S rRNA gene sequencing technique was used in the present study to analyse the characteristics of the oral microbial community at different sites of the peri-implantitis tissues in the beagle dog. The results showed that the dogs’ flora was significantly different from that of humans at the phylum level (e.g. Synergistetes bacteria). By assessing the bacterial com-
munity adhered to the implant surface we observed a high abundance of *Synergistetes*, which showed a significant association with chronic periodontitis. Members of the phylum *Synergistetes* were frequently detected in the human oral cavity at the sites of dental disease, but they have rarely been detected in studies on oral health. Bacteria belonging to the *Synergistetes* phylum mainly live in anaerobic environments, and are considered as opportunistic pathogens; they have been detected in the animal gastrointestinal tract, and human lesions such as abscesses, cysts and periodontal disease. These results suggest that the differences in microbial communities in the peri-implant area between humans and dogs were more significant than previously thought, and thus, extrapolation of the impact of microbes on treated peri-implant diseases in animal models should be done with caution.

**Conclusion**

In this study, we compared the microbial community of submargin group, supramargin group, ligature group and the implant surface of a beagle peri-implantitis model, and found that the residual microbial community of curettes-treated implant surface stayed unchanged. This suggests that the remaining bacteria present on the surface of the implant root proliferates and recovers and affects the treatment result. By using a local drug application or biochemical coating along with the traditional periodontal treatment may improve the treatment efficacy and efficiently restrain the growth of bacteria and prevent biofilm formation.

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**Conflicts of interest**

The authors reported no conflicts of interest.

**Author contribution**

Drs Yu Wei WU, HUI ZHENG, Xue Fen LI and Hui LU contributed equally as first authors. Dr Yu Wei WU and Hui ZHENG designed the project; Drs Hui ZHENG, Hui LU, Yu Wei WU and Xue Fen LI wrote the manuscript; Dr Hui ZHENG produced sequencing libraries and analysed the sequencing data; Drs Hui ZHENG, Yu Wei WU and Xue Fen LI revised the manuscript; Prof. Zhi Hui TANG and Jiu Xiang LIN supervised the overall progress of the project. All authors read and approved the final manuscript.

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