

Comparison of Subgingival and Peri-implant Microbiome in Chronic Periodontitis

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Objectives: To analyse the microbiota composition of 10 healthy dental implants and 10 chronic periodontitis patients.

Methods: Subgingival plaque and peri-implant biofilm were sampled at the first molar site before and after implant restoration. The analysis was conducted by 454-prosequencing of bacterial V1 to V3 regions of 16S rDNA.

Results: Chronic periodontitis subjects showed greater bacterial diversity compared with implant subjects. The relative abundance of sixteen genera and twelve species differed significantly between implant and chronic periodontitis subjects. The genera *Catonella*, *Desulfovibrio*, *Mogibacterium*, *Peptostreptococcus* and *Propionibacterium* were present in higher abundance in chronic periodontitis subjects, while implant subjects had higher proportions of *Brevundimonas* and *Pseudomonas* species.

Conclusions: Our results demonstrate that implant restoration changes the oral microbiota. The analysis suggests that periodontal bacteria can remain for a prolonged period of time at non-dental sites, from where they can colonise the peri-implant.

Key words: 454 pyrosequencing, implant, microbial diversity, periodontitis

Bacteria play a key role in the development of oral disease, including dental caries and periodontal disease¹. Periodontitis is characterised by the formation of an inflammatory infiltration resulting in the destruction of connective tissue attached to teeth, alveolar bone resorption and tooth loss². Periodontitis has a complex bacterial aetiology³ with polymicrobial communities

thought to be involved⁴. It is also influenced by multiple aetiological factors, including biofilm microorganisms, social modulation and genetic elements. Most importantly, periodontitis is associated with bacterial community structures and at least three bacterial species are involved in periodontal disease^{5,6}. Due to early eruptions and unique anatomical structures, first permanent teeth often manifest severely with periodontitis and seem to be refractory during treatment. Several studies have proposed that 'refractory' periodontitis consist of a microbial profile distinct from that of chronic periodontitis which may induce the infection in a susceptible host⁷.

The prevalence of periodontitis in first permanent molars is significantly higher than that in other teeth, whereas the treatment is often ineffective, frequently resulting in the loss of first permanent molars. Dental implants represent a relatively successful treatment strategy for replacing missing teeth⁸. However, implant failure caused by peri-implantitis and peri-implant mucositis has also been reported⁹. To determine the bacterial ecology between disease and health, techniques such as DNA-hybridisation, PCR amplification^{2,10}, and 454 deep-sequencing of 16S rDNA have

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been employed^{11,12}. Deep-sequencing of 16S rDNA allows for the analysis of thousands of sequences per sample, and has been applied to many oral diseases including cavities and colorectal cancer^{13,14}. It is known that bacteria rapidly colonise the peri-implant crevice following implant placement. There were more studies indicating that teeth and implants are affected by different microorganisms due to differences in their structure and composition^{15,16}. Four periodontal pathogens have been identified in the peri-implant sulci of healthy implants of partially edentulous patients: *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola*. These four bacterial strains do not grow around implants in completely edentulous patients¹⁷. The subgingival microflora associated with implants results from the accumulation of saliva or supragingival plaque on the surface of implants¹⁸. Healthy peri-implant sites are characterised by a low ratio of anaerobic/aerobic species and low frequencies for detecting periodontal pathogens^{19,20}. However, there was no information about whether the microbiome of healthy peri-implant biofilm remains similar to that of the subgingival plaque in periodontitis subjects following poor treatment at the same location, or when new microorganisms colonise the implant following removal of the tooth.

The purpose of the present study was to compare the subgingival plaque microbiome of chronic periodontitis patients, whose first permanent molars require removal following basic treatment, with the peri-implant biofilm of healthy implant subjects, who were loaded for 1 year following implantation of the first permanent molars. Furthermore, we investigated whether the microorganisms surrounding the tooth had any changes following removal of the tooth.

Materials and methods

Sample collection and patients

Ten chronic periodontitis patients (CP) were treated by basic treatment (the conventional prophylaxis, supragingival scaling and root planing). Then the patients were measured by clinical assessment to ensure that the periodontitis had an effective control of the teeth except for the first permanent molar, which still had a deeper periodontal pocket with an attachment loss > 2.5 mm. Considering the treatment and the teeth condition, the first permanent molar should be extracted. Ten healthy implant patients (IT) were also recruited, all of whom the first permanent molar was replaced by an implant due to

severe periodontitis. Before implantation, the patients underwent full periodontal therapy (the conventional prophylaxis, supragingival scaling and root planing) until the condition of the other teeth was relatively good. There was no other tooth loss in all samples. No other differences were found in the two groups. All the subjects were selected from the Third Dental Center, Peking University School and the Hospital of Stomatology, Beijing, China. Informed consent was obtained from each patient and the study was approved by the Peking University Biomedical Ethics Committee (Beijing, China). All subjects were non-smokers, non-diabetes, non-pregnancy, non-HIV infection, non-systemic illness, who had not used immunosuppressant medications, anticoagulants or steroids, antibiotic therapy or oral prophylactic procedures within the last 3 months, and did not suffer from other oral diseases such as candidosis, oral lichen planus and leukokeratosis. After removal of supragingival biofilm, the subgingival plaque samples were collected at the first permanent molar before tooth extraction and peri-implant biofilm were collected at the same location after implantation using a standard sterile periodontal probe. In each patient, microbial sampling was performed at about 7 mm probing depth of chronic periodontitis subjects at the first permanent molar and 2 mm probing depth of healthy implants. Plaque samples were then placed in a 1.5 ml sterile tube containing 100 µl of phosphate-buffered saline. They were frozen at -80°C prior to sample processing.

DNA isolation

The bacterial genomic DNA was extracted from the twenty selected samples using QiaAmp DNA mini kit (Qiagen, Hilden, Germany) based on the manufacturer's instructions. The final concentration of DNA samples was adjusted to 20 ng/µl and DNA quantity (A260/A280) was measured using a NanoDrop 8000 spectrophotometer before sequencing (Thermo Fisher Scientific, Wilmington, USA).

PCR primer design and pyrosequencing

The V1 to V3 regions of the primers of the bacterial 16S rDNA were designed to perform pyrosequencing using 454 GS FLX Titanium platform (Roche Applied Science, Indiana, USA). The forward primer was 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer²¹ was 5'-TTACCGCGGCTGCTGGCAC-3'. Unique 10-base pairs barcode sequences were tagged in DNA samples in order to distinguish single samples, allowing for one base mismatch. The Polymerase Chain



Reaction (PCR) reaction procedure was as follows: the initial denaturation at 95°C for 5 min; denaturation at 95°C for 30 s, annealing at 53°C for 30 s, elongation at 72°C for 30 s processing in 22 cycles and the final extension at 72°C for 5 min. The PCR reaction products were purified using the QIAquick Gel extraction kit (Qiagen, Hilden, Germany). Sequencing was performed using homogenous amplicons which were quantified by Qubit Fluorometer (Invitrogen, California, USA). The integrity of samples was tested by Agilent 2100 bioanalyzer (Agilent Technology, California, USA) and qPCR by the AB7500 Real-time PCR System (Applied Biosystems, New York, USA). The raw data were filtered and screened by barcodes and the quality of sequences. Adaptor sequences were trimmed using many factors including gap, mismatch, length of sequences and quality of bases. Sequences with one mismatch, less than two mismatches to the primers, sequences with more than 200 bp but less than 600 bp, or sequences which contained less than six nucleotide mononucleotide repeats were retained. The high-quality sequences were used for further analysis.

Bioinformatics analysis

The selected high quality sequences were assigned to each sample with different barcodes and they were subjected to a BLAST search using the ribosomal database project (RDP), using the MOTHUR (version 1.27) software and then clustered into various operational taxonomic units (OTUs)²². The peri-clustered tags that had 97% similarity were assigned to the same operational taxonomic unit which noted the same taxa level using MOTHUR. Based on the results of OTUs at 97% similarity and the number of tags per sample, Alpha diversity containing richness estimators (Chao and Ace) and diversity estimators (Shannon and Simpson) were analysed using QIIME with default parameters. Rarefaction curves were obtained using those observed sequences and the OTU cluster per sample was based on MOTHUR. Based on the OTU-level classification, the heat map was constructed by using the Bioconductor from R (<http://www.r-project.org>)²³. The relative abundances of OTUs and taxa per sample were calculated based on the number of tags assigned to the same taxa level using LefSe, and Wilcoxon Rank Sum Test for multiple comparisons was carried out between the two groups. According to the matrix of distance, a principal component analysis (PCA) was performed and visualised using the R (<http://www.r-project.org>).

Table 1 The summary information and clinical characteristics of the studied samples.

Characteristics	Implant (n = 10)	Chronic Severe periodontitis (n = 10)
Male/female	3/7	6/4
Age (years ± standard deviation)	42.6 ± 3.6	46.0 ± 3.5
PD (mm ± standard deviation)	2.0 ± 0.9	7.0 ± 2.6
CAL (mm ± standard deviation)	0	7.20 ± 0.78
BL (mm ± standard deviation)	0	6.0 ± 2.5
PB (+/-)	-	+

PD: probing depth; CAL: clinical attachment levels; BL: bone loss; PB: bleeding after probing.

Statistical analysis

Data were analysed for every patient separately. The wilcoxon signed-rank test and fishers exact test and false discovery rate (FDR) test were performed using SPSS (California, USA) for Windows. Significant differences were confirmed by $P < 0.05$.

Results

Pyrosequencing

The basic characteristics and clinical metrics of the first permanent molar in the selected subjects are listed in Tables 1 and 2. The initial and high-quality sequences were obtained from healthy implant subjects and chronic periodontitis subjects. Following quality control and filtering, the number of high-quality tags per sample ranged between 4203 and 22507 sequences. The average number of sequence reads was 11174 for implant subjects and 9747 for chronic periodontitis subjects. More detailed information is provided in Table 3. The microbial diversity of subgingival plaque and peri-implant biofilm in chronic periodontal disease subjects and implant subjects were compared by means of Chao, Ace, Shannon and Simpson indices. Consistent with previous reports, a higher Shannon index and lower Simpson index were found in chronic periodontitis subjects compared with healthy implant subjects, suggesting that the former exhibited significantly greater bacterial diversity.

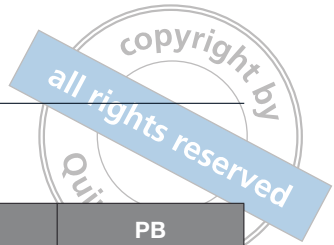


Table 2 The detailed characteristics of the studied samples.

Subjects	Gender	Age (year)	PD (mm)	BL (mm)	CAL	PB
CP1	Male	50	8	5	7	+
CP2	Female	52	7	5	7	+
CP3	Female	51	9	6	8	+
CP4	Female	44	6	6	8	+
CP5	Female	37	7	5	6	+
CP6	Female	52	9	6	8	+
CP7	Female	36	7	6	7	+
CP8	Female	39	8	5	6	+
CP9	Male	42	9	7	7	+
CP10	Male	44	10	7	8	+
IT1	Female	43	2	0	0	-
IT2	Male	42	3	0	0	-
IT3	Female	44	2	0	0	-
IT4	Female	45	2	0	0	-
IT5	Male	37	3	0	0	-
IT6	Female	43	2	0	0	-
IT7	Female	46	2	0	0	-
IT8	Female	47	3	0	0	-
IT9	Female	43	2	0	0	-
IT10	Male	36	3	0	0	-

PD: probing depth; CAL: clinical attachment levels; BL: bone loss; PB: bleeding after probing; CP: chronic severe periodontal disease subjects; IT: implant individuals with healthy peri-implants.

Table 3 The estimates of sequences and the alpha diversity analysis.

Samples	Tags	OTU ^a	Chao	Ace	Shannon	Simpson
Implant (n = 10)	11174 + 3521	207 + 71	291 + 92	324 + 105	3.20 + 0.65	0.12 + 0.08
Chronic severe periodontitis (n = 10)	9747 + 4711	266 + 89	359 + 133	377 + 154	3.88**+ 0.34	0.052*+ 0.020

OTU^a: operational taxonomic unit; each date represents the mean value of ten samples.
 *P < 0.05; **P < 0.01.

The mean values per sample are listed in Table 3. To determine the coverage of our results, a rarefaction curve with a 97% cut off value was plotted for the two groups. The curve demonstrated that the depth of sequencing covered almost all of the phylotypes in the selected samples from Figure 1.

The subgingival and peri-implant microbiomes

The sequences were classified into different OTUs to assess the microbiome composition at various taxonomic levels. Taxonomic composition at the phylum level is shown in Figure 2. The main phyla in 20 samples were *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Actinobacteria*, *Proteobacteria*, *Spirochaetes* and TM7 (uncultured), which accounted for more than 90% of all detected phyla. The periodontitis-associated microorganisms *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola* were detected with low relative abundance in the crevice of peri-implants.

Given that the bacterial microbiome could be altered by the implant, the subgingival and peri-implant microbiomes were analysed based on the OTUs in Figure 3. The three most abundant OTUs associated with the implant were *Pseudomonas*, *Leptotrichia hongkongensis*, *Granulicatella adiacens* and the nine most prevalent periodontitis-associated OTUs related to *Veillonellaceae*, *Parvimonas*, *Selenomonas sputigena*, *Fusobacterium unclassified*, *Bacteroidetes*, *Prevotella loescheii*, *Actinomyces*, *Streptococcus*, *Comamonadaceae*, which were shown in Figure 3. These prevalent OTUs were detected in all samples in both groups and represented the core microbiome.

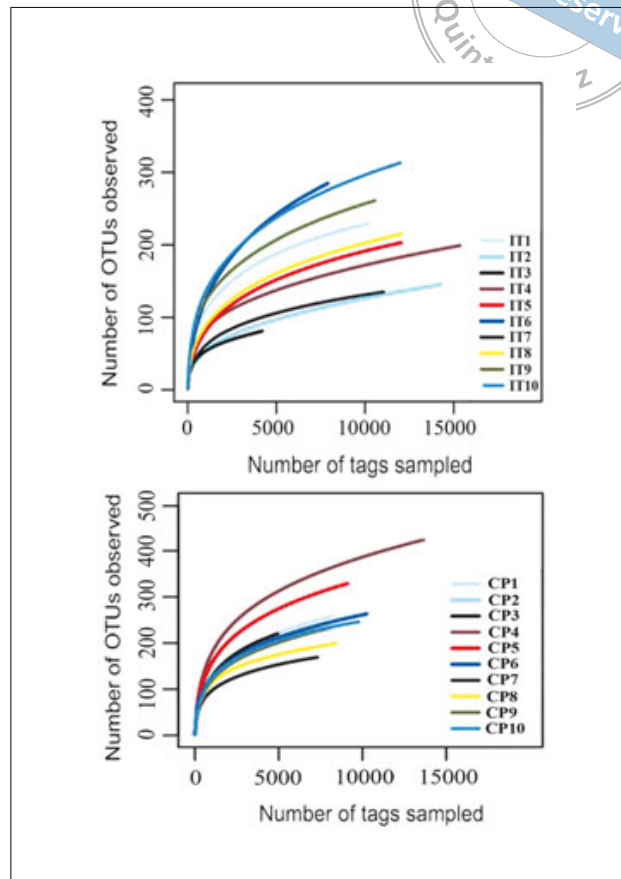


Fig 1 The rarefaction curve of the 20 studied samples.

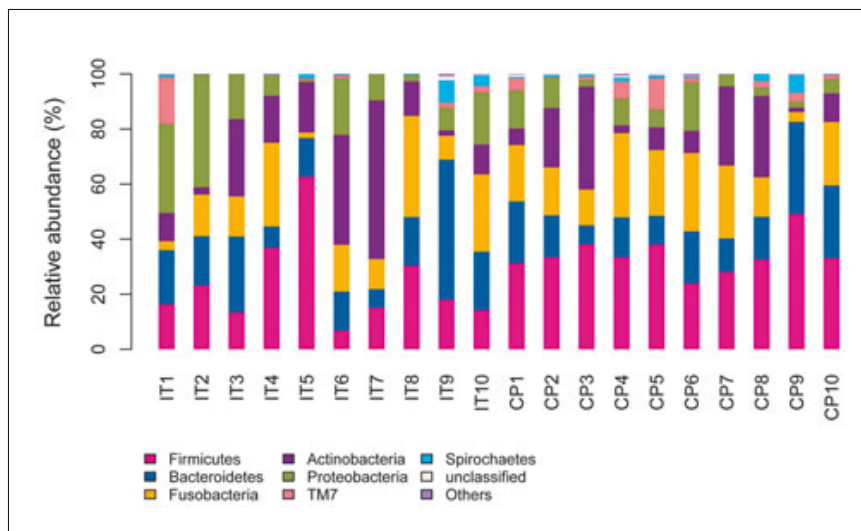


Fig 2 The relative abundance of microbial composition on the phylum level of microbiota of 10 implant patients and 10 periodontitis patients following failed treatment. 'Others' represents some bacteria with a mean relative abundance of < 1% of total sequences.

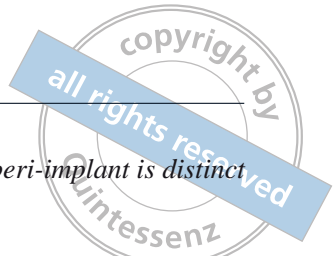


Fig 3 Venn diagram represents all OTUs and unique OTUs from each of the periodontal and implant samples.

The microbial community of the peri-implant is distinct from that of the subgingival

To detect significant differences of the microbiome between the two groups, we compared the relative abundance of various taxa combined with the OTUs. The results showed that sixteen genera and twelve species differed significantly between the implant and chronic periodontitis subjects (Fig 4). Analysis at the genus level indicated that *Pseudomonas* was present in greater abundance in implant subjects compared with chronic periodontitis subjects by 100-fold. The relative abundance of *SRI* genus, *Brevundimonas*, *Catonella*, *Desulfovibrio*, *Mogibacterium*, *Peptostreptococcus*, *Propionibacterium* and *Pseudomonas* differed between the two groups. Accordingly, we performed a community distance metrics analysis to investigate group differences. Implant subjects tended to cluster separately from chronic periodontitis subjects (Fig 5). Several healthy implant subjects displayed negative values for the main elements, whereas disease groups were fixed in the positive values on the first axis. Heat map cluster analysis using the various OTUs yielded similar results. Fourteen OTUs were key elements in the differentiation of chronic periodontitis subjects from implant subjects (Fig 6).

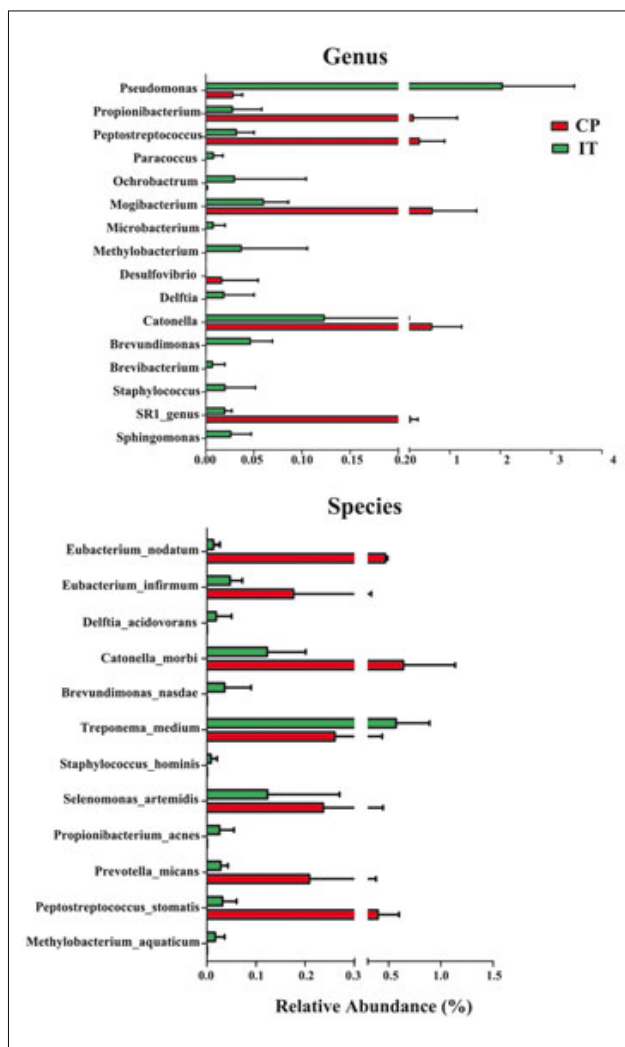


Fig 4 The relative abundance of microbiome analysis of the genus and the species levels between implant patients and periodontitis patients following failed treatment. These bacteria have been presented with $P < 0.05$ by t-test and ANOVA between the two groups.

Discussion

There are hundreds of uncultivated bacterial species in the human oral cavity, and thus the role of the oral microbiome in health and disease is not yet fully understood²⁴. More and more studies have focused on the pathogens of teeth and implants to explore the influence of implants on the oral microbiome using unique technology²⁵. But whether implantation can serve as a good treatment option for tooth loss in severe periodontitis still requires investigation. Implants rapidly influence the bacterial microenvironment. The present study provides new insight into the microbial taxa of the subgingival plaque in chronic periodontitis subjects following failed treatment and that of peri-implant biofilms in the same location in healthy implant subjects.

Higher abundance of periodontal pathogens might colonise clinically healthy sites in periodontitis patients, leading to a higher risk of periodontal disease progression²⁶. This hypothesis may also apply to healthy implant patients. Structural differences between implants and teeth influence the response of the bacterial community as the host response²⁷. The periodontal pathogen bacteria causing periodontitis may have different responses to the peri-implant disease. The genus *Streptococcus* is closely associated with

oral health¹⁷, especially in peri-implants. *Streptococcus mutans* and *Streptococcus anginosus* show higher relative abundance in the periodontitis subjects, while the relative abundance of *Streptococcus salivarius* and *Streptococcus sanguinis* increases responses to the implant. This suggests that the various *Streptococcus* species perform different functions in implant and periodontitis patients. Implants may harbour microbial populations that include some periodontitis-associated taxa, because of the influence of surface energy or the surface structure of implants^{27,28}. The same microbial taxa in the subgingival plaque of periodontitis subjects can also survive in the same location of implants.

Different dental sites are subject to various degrees of periodontal disease. Therefore, the most appropriate implantation program should be selected on a case-by-case basis. The genus *Pseudomonas* is a Gram-negative aerobic gammaproteobacteria that can export numerous proteins relevant to the pathogenesis of clinical strains²⁹. The relative abundance of two phyla, five classes, nine orders, seventeen families, sixteen genera and twelve species differs significantly between healthy implant subjects and chronic periodontitis subjects following failed treatment. This suggests that healthy implants do not influence individual genera or species but rather the composition of the entire microbial community. The oral microbial flora is the major source of bacteria that colonises implants. A higher ratio of pathogenic bacteria has been reported in implant patients compared with periodontitis patients²⁰. Moreover, it appears that Gram-negative anaerobic bacteria rapidly colonise

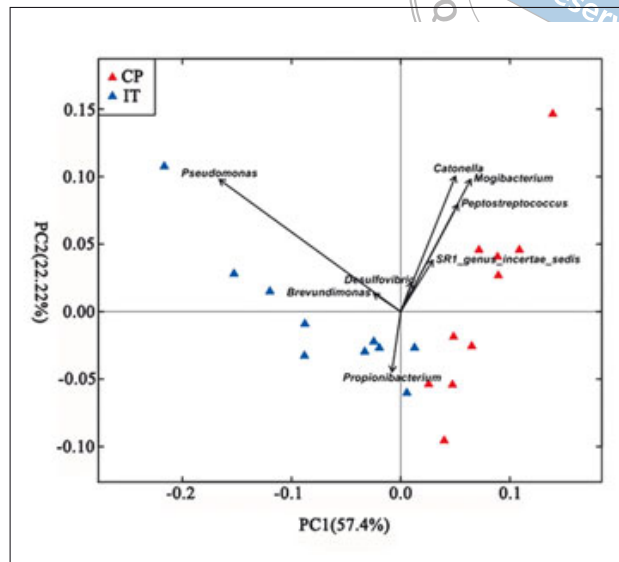


Fig 5 The principle component analysis of the bacterial community between 20 samples based on the different genera. The periodontitis patients following failed treatment, cluster away from the implant patients. The 10 implant samples were coloured blue, whilst the 10 periodontitis patients following failed treatment were coloured red.

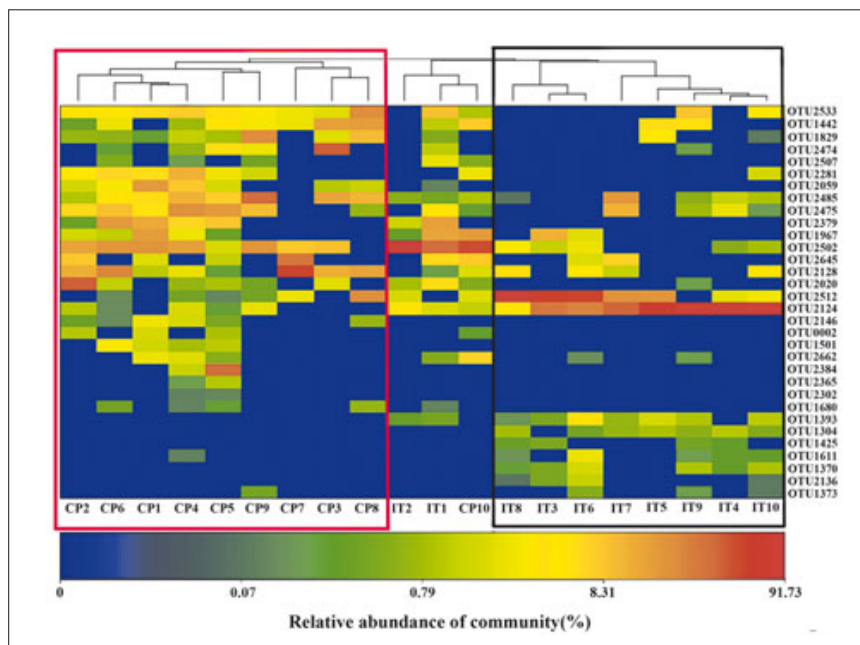
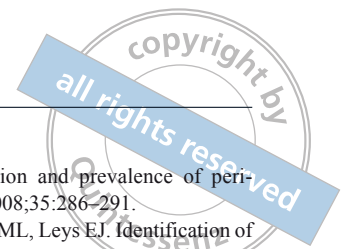


Fig 6 The heat map and cluster analysis of the different OTUs between 10 periodontitis patients following failed treatment and the 10 implant patients. Thirty-two different OTUs were analysed using the relative abundance. In the heat map, areas where it is dark indicate a higher relative abundance of these OTU.



the peri-implant sulcular. Although *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola* are the most frequently detected pathogens in periodontitis; they also appear in peri-implant sulcular. Therefore, the health status of the patients should be monitored carefully before implantation³⁰. The microbial composition of the adjacent subgingival biofilm of implant patients should also be assessed, because it influences the expression of gingival crevicular fluid biomarkers²⁶.

Conclusion

We examined the bacterial composition of subgingival plaque in chronic periodontitis patients following failed treatment, and the peri-implant biofilm of healthy implant patients in a similar location. Our results provide a new insight into the responses to implantation therapy from patients who responded poorly to basic periodontal treatment. Subgingival biofilms exhibited higher bacterial diversity compared with peri-implant biofilms. Implantation alters the composition of microbiome. Moreover, the periodontal bacteria can survive at non-dental sites for a long period of time, from which they further partly colonise the peri-implant sulcular.

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